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Reason, in itself confounded,
Saw division grow together;
To themselves yet either neither,
Simple were so well compounded,

That it cried, 'How true a twain
Seemeth this concordant one'!

—SHAKESPEARE,
The Phœnix and the Turtle.

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Page 959, *table 4, continued. For first subtitle* 'Healthy' Males *read* 'Healthy' Females, continued.

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ENDOCRINOLOGY

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GONADOTROPIC HORMONE

ASSAYS OF HUMAN MALE URINE[1]

EMILY J. HELLER, CARL G. HELLER
AND ELMER L. SEVRINGHAUS

From the Department of Medicine, University of Wisconsin Medical School
MADISON, WISCONSIN

ATTEMPTS are being made through the assay of gonadotropic hormone in human urine to furnish an aid in the clinical diagnosis and treatment of gonadal dysfunctions, as well as to add further information on the role of this hormone in the relationship between the pituitary and the gonads. Although even the best assay methods are only roughly quantitative, and many of the findings probably misleading and unreliable, sufficient progress has been made in recent years to justify the hope that such studies may eventually leave the research laboratory, and enter the field of practical application.

We have been able to apply greatly improved extraction and assay technics for gonadotropic hormone to routine analyses of urine. A study of the excretion rate of this hormone in a series of women of various ages revealed that urinary gonadotropic concentration was low in menstruating women, increased markedly at the menopause, and remained consistently high during the post-menopausal period and senility (1). It seemed of interest to conduct a similar study in a series of normal men of various ages.

Surprisingly little attention has been paid to the excretion of gonadotropic hormone in the urine of men. The study has been limited mainly to the determination of large quantities of anterior pituitary-like hormone in cases of teratoma testis, (2, 3), and the hormone of male castrates (4, 5). The consistent presence of gonadotropic hormone in the urine of normal men has often been denied (6). Except in cases of teratoma testis, several workers, including Ferguson (7) have reported negative findings in large series of cases. Osterreicher (8) found that in 33 men 20 to 30 years of age, no hormone was present in amounts sufficient to show activity in the ranges for which the test was designed. Urines of 53 men 50 to 91 years old gave 11 positive

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responses. Saethre (9) was able to demonstrate the presence of varying amounts of gonadotropic hormone in the urine of about half the cases he studied.

Katzman (10), Friedman and Weinstein (11), Leathem and Morrell (12) and McCullagh (13) have also been able to show the presence of gonadotropic activity in extracts of normal male urine.

MATERIALS AND METHODS

Complete 12-hour night specimens of urine were collected from 41 male adults ranging in age from 20 to 95 years. None of these subjects had any obvious metabolic disturbance. Aliquots of total 12-hour samples were precipitated with alcohol; the precipitate dried and washed with ether and taken up in tap water, after removing toxic salts by dialysis through a collodion or cellophane bag. The details of the extraction procedure were identical with those previously described (14), with the exception that, as work progressed, it was found more convenient to reprecipitate

TABLE 1

Subject R.B. Age 25

Daily sample	Aliquot[1]	Uterine weight	Ovarian weight
cc.	cc.	mg.	mg.
Control		21.6	12.0
		(19.6–26.7)[2]	(8.8–14.2)[2]
610	400	52.0	13.8
500	250	30.8	13.6
650	200	39.4	14.0
	400	66.0	18.2
	Estimated titer: 200 cc.		

[1] Volume of urine from which material to be injected was derived.
[2] Ranges of control data.

the material than to evaporate the dialyzed solutions down to the desired volume. The final solutions, which represented given fractions of the total 12-hour specimens, were injected into 24-day-old rats in 1.0 cc. amounts twice daily for 3 days; autopsies were performed on the fourth day. Uterine and ovarian weights were used as assay endpoints (15). The minimal volume of urine necessary to produce minimal uterine weight increase was estimated from the response to the known equivalents of the original urine volume injected. From 400 cc. to 100 cc. aliquots of the 12-hour specimen were necessary to effect minimal stimulation in the assay animals. The minimal volume of urine which produces minimal uterine weight increase is referred to as the titer of the urine specimen. The titer is merely a simple and convenient term to designate the estimated amount of gonadotropic hormone present in a given urine sample. Since in the majority of cases, it was necessary to inject the equivalent of most of the total volume of a sample into one assay rat, in only a few instances was it possible to make an accurate assay curve for each daily sample. It was therefore expedient to assign an 'average titer' to each subject from the results of three or more daily samples. An example of how the titers were estimated is given in table 1. It should be emphasized that these titers are only approximations, and also that they are only the estimated titers for the particular days each subject was studied.

RESULTS AND DISCUSSION

Gonadotropic hormone was present in the urine of all normal men in sufficient quantities to be detected daily. In order to determine what variation to expect from

day to day in the same individual, 10 of the subjects (20 to 75 years old) collected specimens nightly over periods from 10 to 40 days. The titer of each subject was found to vary considerably from day to day. Although variations from one day to the next often ranged as high as 100%, the deviation of the individual daily titer from the average titer rarely exceeded 35% regardless of the size of the average titer. These variations in daily excretion are probably of no physiological significance, and merely indicate that the male excretion rate over a wide age range is somewhat erratic. Some of the variation may undoubtedly be assigned to the technic of extraction and the response of the assay animal. In no case could any tendency towards a periodic cyclic increase or decrease in excretion be demonstrated. Figure 1 illustrates the amount of daily variation in one of the subjects.

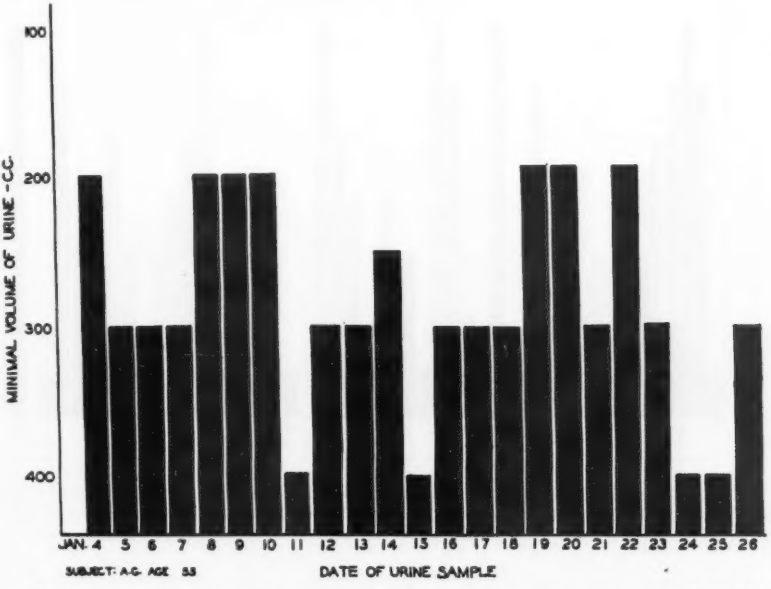

Fig. 1. Daily variation in gonadotropic hormone output for a normal male. The minimal volume of urine—cc. indicates the least amount of urine that had to be concentrated in order to stimulate uterine development in the rat.

An age classification of the series reveals that the range of titers in each age group approximates the range of titers for the entire series (fig. 2). For instance, specimens from some of the younger men were just as high in gonadotropic potency as some of the older men, while some of the older men had urinary gonadotropic titers just as low as some of the younger men. In other words, a review of the series shows no consistent increase in the concentration of gonadotropic hormone in the urine with advance in years of the men. These findings are in agreement with those of Saethre (9), who also was unable to find large increases in excretion in elderly and senile men.

A comparison of these results with those obtained in the studies of the excretion rate in the female (1, 16) reveals several striking differences. Urinary gonadotropic concentration is low in all women with regular normal menstrual cycles, whether young girls, or mature women near the climacteric age period. Women with normal cycles generally excrete enough hormone daily to demonstrate gonadotropic activity

if enough urine is concentrated. Their titers vary roughly between 400 and 800 cc. with rises above this level occurring during the intermenstrual period, usually somewhere between the tenth and twentieth day of the cycle. In making comparisons this intermenstrual period was avoided.

Women near the menopause having irregular cycles show a somewhat higher hormone excretion. In menopause or senile women in whom cycles have ceased, the titers are uniformly high, the minimal volume of urine required lying between 25 and 10 cc. Therefore, in the female there is a marked increase in the amount of excretion of gonadotropic hormone at the time of the climacteric, after which the excretion remains at the same high level. It should be pointed out that the term, menopause, refers to

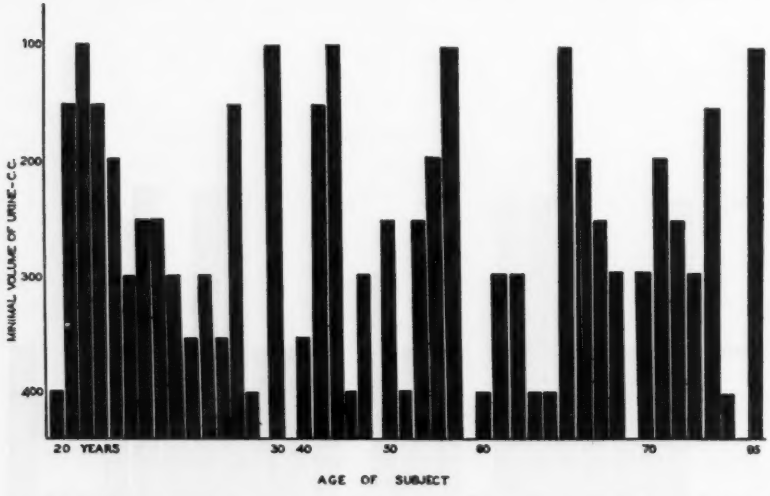

Fig. 2. THE GONADOTROPIC HORMONE EXCRETION OF NORMAL ADULT MALES. The minimal volume of urine—cc. indicates the least amount of urine that had to be concentrated in order to stimulate uterine development in the rat.

women who are observing irregularity and lengthening of cycles or to women whose cycles have ceased. It does not refer to the presence of psychic or vasomotor symptoms; the presence or absence or severity of such symptoms is not correlated with the gonadotropic potency of the urine.

The male shows an erratic, considerable and frequent variation in daily excretion regardless of age, as contrasted with the female who remains quite consistently at the same level of excretion in the menopause or senile age periods, or shows a periodic increase and decrease in potency during each menstrual cycle. Young adult males generally excrete gonadotropic hormone in higher concentrations than do most young females except during the intermenstrual rise. In no instance was the titer of any normal male in any age period as high as that exhibited by the female in the menopause or post-menopause period. The highest titers of the normal males were around 100 cc. aliquots of urine, while those of the females were so high as to require in some cases only 10 cc. of urine to elicit a positive response in the assay rat. The rapid rise and great increase in the potency of the urine in the female as she passes into the menopause does not appear to be duplicated by the male with advance in years. Figure 3

illustrates in diagrammatic form the differences in the range of gonadotropic titers through adult life in males and females.

In 4 cases, titers were comparable to the high potency of the urines in the menopause or senile periods in women. None of these male subjects were normal. Two of them had been diagnosed as involutional, one was eunuchoid, and the fourth had carcinoma of the testes. It is well known that men with malignant tumors of the testes excrete large quantities of gonadotropic substance. Reports in the literature of other cases of undescended testes due to inherent failure of the gonads indicate an increase in gonadotropic excretion. This type of eunuch seems to excrete the hormone in amounts comparable to the level of excretion in male castrates (17). We were unable to obtain any true male castrates, and therefore could not verify these reports.

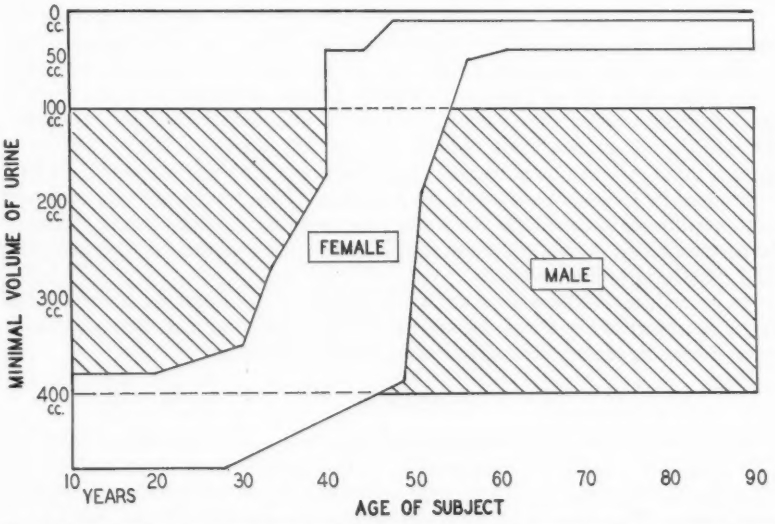

Fig. 3. Comparison of the ranges of gonadotropic titers through adult life in males and females.

The two men with involutional symptoms whose urines exhibited high potency led us at first to believe that probably all such cases had a tendency to excrete more of the hormone. However, since that time, three more males with involutional symptoms whose titers fell into the normal range have been studied. It seems probable, therefore, that as in the case of the females, the presence or absence of psychic symptoms is not correlated with urinary gonadotropic potency.

In cases of tuberculosis of the testes, and in benign growths which are not thought to produce an increase in gonadotropic hormone themselves, the Aschheim-Zondek test may give positive responses (3), so that these cases also seem similar to true castrates. In two such cases studied by us, the gonadotropic titer was similar to that of the menopause woman.

Our findings show that there is considerable difference between the amounts of gonadotropic hormone excreted by elderly males and females. Yet, apparently male castrates, whether castrates by surgery or disease, are similar to castrate or menopausal females in respect to excretion of the hormone. Is it possible that the testes, unlike the ovaries, are still capable of playing a role in the pituitary-gonadal relation-

ship well into old age? Could it be that gonadal tissue, whether male or female, when it becomes inactive or is removed from the body, no longer uses or neutralizes the gonadotropic hormone present in the blood? Seidlin (18) has suggested that the ovaries use gonadotropic hormone, and has shown that greater amounts of injected hormone are excreted by castrated guinea pigs than by intact guinea pigs. Such a theory would fit the accumulated data from this laboratory. The amount of hormone excreted would thus be an index of the amount of hormone used by the gonads. In menopause and castrate women, and in castrate and eunuchoid men the gonads are inactive or absent, no hormone is used, and it therefore accumulates in the blood and is excreted in large quantities in the urine. In menstruating women and in normal men of all ages relatively small amounts are excreted since the gonad stimulating substance of the pituitary is being used by the gonads.

The fact that recession of secondary sex characteristics, common to the post-menopausal woman, is seen rarely in elderly men would support the contention that the testes have some endocrine function in old age. Werner (19) says, "That some few men are capable of fertilization (production of viable spermatozoa) to the advanced age of 80 years and beyond is an accepted fact, but this does not militate against the evidence that many men show beginning decline of potency at about 50 years of age, and during this period in life, they have the typical climacteric syndrome which is mitigated by treatment with male sex hormones." It is possible that the two men with involutional symptoms in our series were experiencing a climacteric similar to the menopausal syndrome, and thus excreted amounts of gonadotropic hormone comparable to the menopause woman.

In general, our findings suggest that in respect to gonadotropic excretion, the male counterpart of the female menopause is uncommon, and that the male gonads are often capable of some form of endocrine function in old age.

SUMMARY

Excretion of gonadotropic hormone was found to occur in a series of normal males of varying ages in sufficient concentration to be consistently demonstrated by the methods used. Daily excretion in the same individuals was variable, but no periodicity was discerned. The amounts of gonadotropic hormone excreted by the normal male of any age ranges between titers of 400 cc. to 100 cc. aliquots of urine. The rise in the concentration of the hormone in the urine of the female at the menopause is not duplicated by the male with advance in years. Since the male castrate, whether castrate by surgery or disease, and the eunuch seem to excrete gonadotropic hormone in amounts comparable to the menopause and castrate woman it is interesting that there is such a large difference in the excretion rates between post-menopausal women and elderly men. It is suggested that the testes in later decades unlike the ovaries, are still capable of playing a role in the relationship between the pituitary and the gonads.

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ADDENDUM

Since this paper was submitted, a report by Dr. Louis Levin (*Endocrinology* 28: 378. 1941) has appeared in which he compared the percentage recovery of gonadotropic hormone by the Heller and Heller modification of the alcohol method with that by his tannic acid procedure. He found that a greater amount of hormone was recovered when using tannic acid precipitation. In his hands, the loss of gonadotropic potency in the alcohol method appeared to take place principally during dialysis and the subsequent manipulation of the extract. Since we were unable to demonstrate a loss of potency when using the method adopted by us for routine work, several points seem worth mentioning. First, Dr. Levin did not follow the alcohol method as described by us. We do not adjust the pH of the urines or extracts. The dry precipitates are eluted with tap water, and are not leached with dilute alkali. We dialyze the solutions no longer than 4 hours, and then reprecipitate them with 4 volumes of alcohol, and not with acetone. Evaporation of the dialyzed solutions was abandoned early in our studies because it was not easy to control and losses occurred if too much evaporation took place. Second, the alcohol method has the advantage of being non-toxic when using extracts derived from large volumes of urine. There have been no deaths, and very few evidences of toxicity in the several thousand animals used in our studies. Alcohol extracts from complete 24-hour urine samples may be injected into one animal without toxic effects, whereas the tannic acid method allows only a fraction of a total 24-hour volume to be injected. When as much as $\frac{1}{5}$ of a 24-hour specimen was used, Dr. Levin reports that 40% of his animals showed definite toxic symptoms or died. The result is that not enough tannic acid extract can be injected to determine the actual base level of excretion when studying low potency urines as in the case of normal young women.

GONADOTROPIC ACTION OF NORMAL MALE URINE EXTRACT ON OVARIES OF NORMAL AND HYPOPHYSECTOMIZED IMMATURE RATS AND OF IMMATURE MICE[1]

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THE PRESENCE of gonadotropic activity in the urine of normal men has been demonstrated by several investigators (1–10). Because of the low concentration of the active substance in the urine and the consequent necessity for injection of large amounts of extract (in urine equivalents), little work has been done to determine the qualitative nature of the ovarian response to such extracts. However, within the last several years an extract, Prospermin, stated to be prepared from normal male urine, has become available and several investigations on the action of this preparation have been reported.

Leathem and Morrell (11) found that in the dog Prospermin produces pronounced follicle stimulation and all the manifestations of estrus. Similar results were obtained with immature female cats, although luteinization was obtained after injection periods of more than 12 days (12).

Wells and Overholser (13) reported that the same extract stimulates sperm formation in mature and immature hypophysectomized ground squirrels, indicating follicle stimulating activity. Comparable results were obtained in hypophysectomized male rats (14). On the other hand, Evans et al. (15) and Jensen and Tolksdorf (16) state that Prospermin contains mainly ICSH with perhaps traces of follicle stimulating activity.

Because the qualitative nature of the response to normal male urinary gonadotropin still remains not clear, it was decided to make a study of the action of such extracts on the ovaries of normal and hypophysectomized rats. Two Prospermin samples were used but since the results obtained with this substance were contradictory to those previously obtained by Tyndale and Levin (17) with male urine gonadotropin, a series of hypophysectomized rats were injected with a preparation of normal male urine made by the method of Levin and Tyndale (18). In addition, the action of the two male urine preparations, as well as one from human pregnancy urine, were compared in intact immature female mice.

The results of these studies indicate that the two preparations from male urine are quite different in their effects on the ovary of the hypophysectomized rat. The Levin-Tyndale extract exerts a follicle stimulating effect very similar to that obtained with castrate or postmenopausal urine gonadotropin. The Prospermin, on the other hand, has very pronounced luteinizing properties and in some respects resembles pregnancy urine gonadotropin. These findings, confirmed by the comparison in immature mice, are presented in this communication.

MATERIALS AND METHODS

In order that subsequent results might be evaluated on the basis of equal doses of

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gonadotropin, three types of preparation, Prospermin KX,[2] *L582A* and *L594B* (prepared from pooled urine of male medical students by the method of Levin and Tyndale) (18), and *L477*, a human pregnancy urine preparation made by the same method, were assayed by the mouse uterus technic (2). A total of 254 mice were used for the assays.

A second Prospermin sample (KK) was not assayed in mice.

The comparisons of the action of the preparations were carried out using 238 immature female rats of which 112 were normal and 126 were hypophysectomized.

The normal rats were 22 days old at onset of treatment and the average body weight was 43 gm. (range, 30 to 50 gm.). These rats were injected with Prospermin KX once daily for 5 days and killed on the sixth day, 120 hours after the first injection. All injections were made subcutaneously.

Most of the hypophysectomies were performed on immature female rats 30 to 33 days old, a few being operated as early as the 26th day and as late as the 38th day of life. Only animals in which the vaginae were closed at the time of operation were used. Body weights at operation averaged 69 gm. (range, 50 to 94 gm.) and at autopsy, 10 days later, averaged 64 gm. (range, 48 to 85 gm.). A postoperative interval of 5 days was uniformly allowed to elapse before treatment was instituted. Daily subcutaneous injections were then given for 5 days and the animals killed on the 6th day. The Prospermin was injected once daily whereas the daily dose of preparation *L594B* was divided into 2 injections.

At autopsy, the ovaries, adrenals and uteri were dissected out and weighed, the latter after removal of intra-uterine fluid. Pituitary capsules were serially sectioned, stained with Masson trichrome and examined microscopically for pituitary fragments. Data from animals retaining pituitary fragments, no matter how small, were discarded.

All ovaries were examined for signs of luteinization under the binocular dissecting microscope at the time of autopsy. In addition, the ovaries of all the normal rats receiving the smaller doses of Prospermin were sectioned serially and examined microscopically. The larger doses of Prospermin produced ovaries largely lutein in nature. This was substantiated by examination of serial sections of at least half of the ovaries from rats on each of these doses. The ovaries of all hypophysectomized rats were microscopically examined after serial sectioning.

RESULTS

Mouse assays. The results obtained from the mouse assays show that there is a great difference in the potency of the three preparations used (table 1). The mouse uterus unit is 1.25 γ, 10.0 γ and 2.5 mg., respectively, for *L477* (PU),[3] Prospermin KX and *L582A* (from normal male urine).

The very similar uterine weight curves obtained with the three preparations (fig. 1) indicate that a comparison on the basis of doses determined in this way is valid. It may be seen that the ovarian weight curves for the Prospermin KX and for PU (*L477*) are almost identical (fig. 1), showing the small increase in weight with increased dosage which has been observed previously with pregnancy urine gonadotropin. In contrast, the ovarian weight curve for *L582A* was quite different from the other two and closely resembles that obtained with preparations from the urine of castrate or postmenopausal women. The increase in ovarian weight was much greater

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[2] Normal male urine extract, in the form of Prospermin, was furnished through the kindness of Dr. J. A. Morrell of E. R. Squibb & Sons.

[3] Gonadotropin from the urine of ovariectomized or postmenopausal women is designated as CU. Gonadotropin from human pregnancy urine is designated as PU.

at every dose level tried, *L582A* producing ovaries about twice as heavy as those resulting from the Prospermin or the PU.

The luteinizing abilities of the three preparations also showed an interesting difference. As indicated in table 1, Prospermin produced neither hemorrhagic follicles nor corpora lutea, as revealed by gross examination under the dissecting microscope,

TABLE 1. GONADOTROPIC ASSAY. SWISS MICE

Total dose		No. mice	Mean body wt.		Organ weight $\pm \Sigma_m$[3]		Vagina swollen or open	B.P. C.L.
			Start	End	Ovaries	Uterus		
γ	M.U.U.[1]		gm.	gm.	mg.	mg.	%	%
Prospermine KX								
5.0	0.5	6	9.8	10.7	2.3±0.2	6.3±0.2	0	0
10.0	1	13	10.2	10.6	2.4±0.1	13.7±2.0	50	0
20.0	2	14	10.4	10.5	2.5±0.1	34.4±3.3	100	0
30.0	3	10	9.1	10.1	2.4±0.2	29.0±3.7	90	0
50.0	5	12	9.8	10.2	2.8±0.1	35.3±2.2	100	0
100.0	10	12	10.1	11.2	3.5±0.2	28.3±3.3	100	67
250.0	25	6	10.6	11.7	4.7±0.3	21.7±3.2	100	83
1000.0	100	6	10.6	11.7	5.8±0.3	27.5±2.4	100	100
Normal Male Urine L582A								
2.0[2]	0.8	8	9.7	11.2	3.4±0.4	10.4±1.3	50	0
2.5	1	8	9.4	11.0	3.3±0.2	16.4±3.9	50	0
3.0	1.2	8	10.3	11.7	3.4±0.2	23.5±4.1	88	0
4.0	1.6	8	10.8	12.1	4.5±0.2	38.1±2.1	100	0
5.0	2.0	11	10.8	12.5	4.6±0.4	35.9±1.9	100	0
10.0	4	9	10.3	12.7	7.1±0.5	42.0±2.5	100	0
20.0	8	9	9.9	12.3	8.0±0.9	34.1±4.4	100	0
60.0	24	3	10.5	11.4	9.2±0.8	25.0±2.3	100	*
Human Pregnancy Urine L477								
0.63	0.5	8	10.6	11.4	2.7±0.2	7.4±0.5	0	0
1.25	1	8	11.5	12.4	2.8±0.2	14.5±2.5	50	0
2.5	2	16	11.4	12.8	3.0±0.1	30.6±3.4	75	0
5.0	4	15	10.8	12.4	3.3±0.2	39.5±3.4	100	0
10.0	8	12	10.9	12.3	3.9±0.2	31.1±4.3	83	8
25.0	20	9	11.0	12.5	4.8±0.3	18.1±0.4	100	88
50.0	40	8	10.1	11.0	5.0±0.3	20.6±1.4	75	50
100.0	80	4	11.6	12.3	6.0±0.5	22.5±0.9	75	50
200.0	160	4	10.4	12.0	5.9±0.7	23.7±1.3	100	75
Controls		27	10.6	11.2	2.7±0.1	6.3±0.4	0	0

[1] M.U.U. = mouse uterus units.
[2] The doses of *L582A* are given in milligrams.
[3] Σ_m = Mean deviation of the mean.
* 1 ovary of 1 mouse showed a very small hemorrhagic point.

at the 5 M.U.U. (50 γ) dose level. However, when 10 M.U.U. were administered, the ovaries of 67% of the mice grossly showed blood points or corpora, chiefly the former. It is possible that microscopic examination of the ovaries of the mice on the lower doses would have revealed luteinizing tendencies. With the PU preparation (*L477*), blood points and corpora were seen grossly in the ovaries of 8% of the mice receiving 8 M.U.U. and in 88% of those receiving 20 M.U.U. The luteinizing and hemorrhagic follicle forming abilities of these two preparations thus seem to be in good agreement.

In contrast to these results are those obtained with the male urine extract prepared by us. No blood point formation or luteinization was seen in the ovaries of any of the mice treated with this preparation even though the ovarian weights elicited were much larger than those produced by the PU or Prospermin.

Normal rats. Groups of 7 to 11 normal 22-day-old female rats were injected with 12 different doses of Prospermin KX (table 2). The lowest dose administered, 0.025

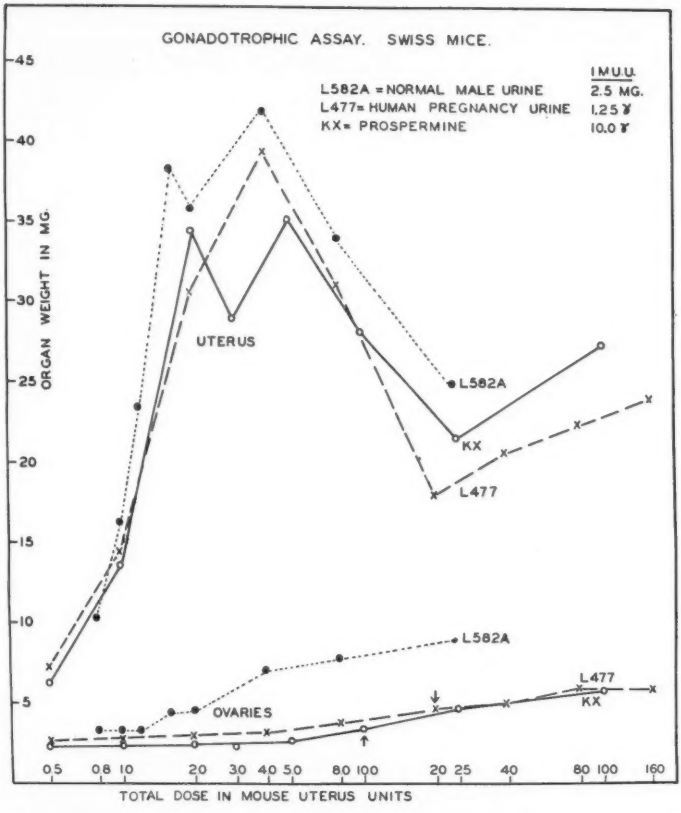

Fig. 1. Comparative assay of the gonadotropic potency of pregnancy urine (*L*477), Prospermin KX and normal male urine (*L*582*A*).

mg., had no effect on ovarian weight or histology. Increasing the total dose to 0.065 mg. resulted in some increase in ovarian weight. In the ovaries of 6 of the 10 animals receiving this dose only small and a few medium size vesicular follicles were observed. Evidence of thecal luteinization was slight. However, the ovaries of the other 4 animals on this dose showed thecal luteinization as well as the presence of 1 to 3 large corpora lutea. There were no large normal vesicular follicles. Ovarian weight was greatest in the presence of lutein tissue.

Ovarian weight was further increased by increasing the dose to 0.125 mg. Only one of the 9 animals exhibited corpora lutea and another had a single hemorrhagic

follicle. In general the ovaries were composed of small follicles and a few medium-sized vesicular follicles. The injection of 0.250 mg. did not cause further increase in ovarian weight but did further increase the luteinizing action as 5 of the 7 rats had large corpora lutea in addition to a few stimulated follicles. All animals that received a total dose of 0.5 mg. or more of this extract exhibited corpora lutea. These relatively heavy ovaries consisted of large vesicular follicles, large follicles with thickened gran-

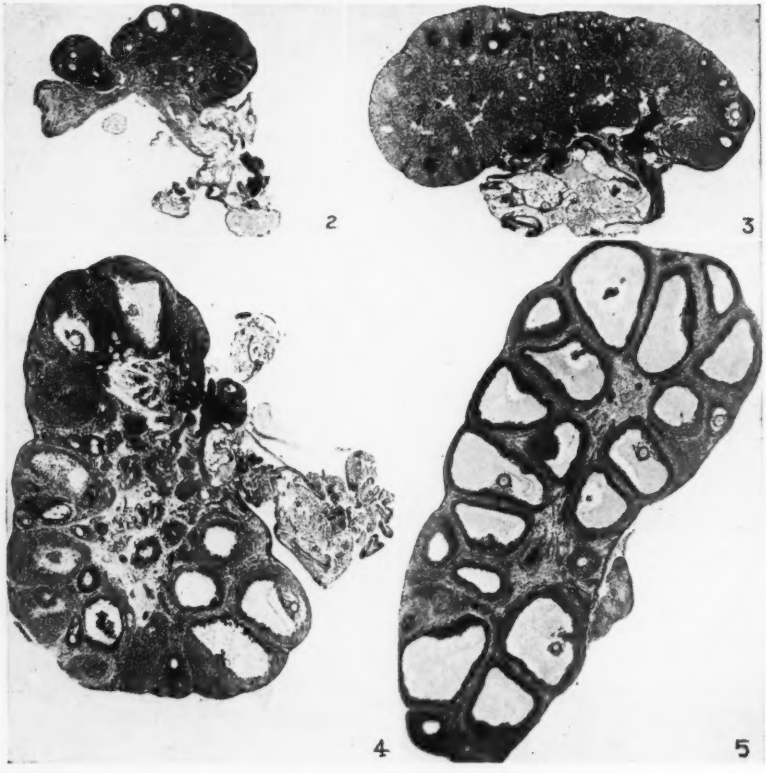

Fig. 2. OVARY OF AN UNTREATED HYPOPHYSECTOMIZED RAT. Weight of both ovaries 6.0 mg. ×70
Fig. 3. OVARY OF AN HYPOPHYSECTOMIZED RAT FOLLOWING THE INJECTION OF PROSPERMIN. Total dose 2.5 mg. Ovarian weight 23 mg. Ovary primarily composed of luteinized thecal tissue. ×70. Fig. 4. OVARY OF AN HYPOPHYSECTOMIZED RAT FOLLOWING THE INJECTION OF PROSPERMIN. Total dose 30 mg. Ovarian weight 77 mg. Most follicles exhibit granulosa luteinization. ×70. Fig. 5. OVARY OF AN HYPOPHYSECTOMIZED RAT FOLLOWING THE INJECTION OF 144 MG. OF MALE URINE EXTRACT *L594B*. Ovarian weight 75 mg. Only large vesicular follicles are present. ×70.

ulosa, and corpora lutea. Many corpora lutea atretica were observed. Maximum ovarian stimulation was attained at the 20 to 25 mg. dose levels.

Hypophysectomized animals. Groups of 4 to 10 hypophysectomized rats were treated with Prospermin KX at 7 different dose levels (table 3). Ovarian weight was significantly increased with all doses but the average ovarian weight was not markedly different with doses ranging from 2.5 to 20 mg. The uterine weight progressively increased with the dosage.

TABLE 2. RESPONSES OF 22-DAY-OLD FEMALE RATS TO PROSPERMIN KX

Total dose		No. rats	Mean body weight		Organ weights	
mg.	M.U.U.		Start gm.	End gm.	Ovaries mg.$\pm\Sigma_m$[1]	Uterus mg.$\pm\Sigma_m$[1]
0.000	0.0	16	42.0	50.0	12.4±0.3	34.0± 1.1
0.025	2.5	9	46.9	57.7	11.9±0.6	41.6± 3.6
0.065	6.5	10	41.3	57.3	18.6±1.6	100.5± 9.1
0.125	12.5	9	41.0	52.8	21.0±1.5	116.3± 5.1
0.250	25.0	7	46.3	55.8	21.1±2.5	90.6±12.7
0.500	50.0	7	40.4	54.8	29.8±6.7	77.1±10.3
1.0	100.0	9	45.1	60.6	41.6±8.7	80.2± 6.3
5.0	500.0	11	39.1	54.6	61.7±5.8	123.8± 8.0
10.0	1000.0	7	38.6	47.8	63.6±4.9	147.7± 5.8
15.0	1500.0	7	39.6	49.9	61.6±5.9	129.1± 9.7
20.0	2000.0	7	40.5	50.0	70.3±6.6	142.0±10.3
25.0	2500.0	7	44.0	56.2	92.3±5.7	124.8± 8.7
30.0	3000.0	7	37.8	46.5	74.0±4.4	122.4± 7.1

[1] Mean deviation of the mean.

The two lowest doses (2.5 and 5 mg.) failed to stimulate follicles in the ovaries but instead caused considerable thecal luteinization. Only a few normal vesicular follicles were present (fig. 3). With 10 and 15 mg. doses the general effect observed was thecal luteinization. However, the stimulation of 2 or 3 follicles to medium-large size was evident in several of the ovaries, whereas corpora lutea atretica were also observed in one case. Stimulation of several follicles to large size and thecal luteinization, in the absence of corpora lutea, was observed with one rat which had received 20 mg. Ovaries of other animals on the same dosage contained many corpora lutea atretica. The largest doses (25 to 30 mg.) produced large vesicular follicles and corpora lutea atretica with varied proportions of these structures being found (fig. 4).

TABLE 3. RESPONSES OF HYPOPHYSECTOMIZED IMMATURE FEMALE RATS TO PROSPERMIN

Total dose		No. rats	Mean body weight		Organ weights	
mg.	M.U.U.		Start gm.	End gm.	Ovaries mg.$\pm\Sigma_m$[1]	Uterus mg.$\pm\Sigma_m$[1]
			Prospermin KX			
0.0	0.0	9	77.1	67.2	6.9± 0.3	21.0± 0.5
2.5	250.0	4	60.8	61.8	23.4± 1.0	32.5± 0.8
5.0	500.0	7	70.3	65.7	20.8± 1.6	62.5±10.3
10.0	1000.0	10	76.9	67.9	28.2± 2.0	71.6± 5.8
15.0	1500.0	7	66.9	60.6	22.8± 2.0	79.7±17.1
20.0	2000.0	7	70.9	65.7	30.1± 2.3	117.0±15.6
25.0	2500.0	6	75.2	69.8	39.3± 8.3	112.0±10.9
30.0	3000.0	4	77.5	65.3	77.0±21.0	143.5±12.2
			Prospermin KK			
2.5		6	60.3	61.0	9.7± 1.0	39.0± 6.6
5.0		8	61.3	58.4	12.1± 0.1	42.0± 5.8
10.0		8	66.8	66.0	20.3± 0.5	64.0±10.1
15.0		3	64.2	62.3	27.0± 4.6	78.0± 9.4
20.0		4	67.0	60.3	73.8±12.6	129.0± 4.5
25.0		6	74.0	69.2	96.3±11.8	138.0± 3.9
30.0		2	84.0	69.5	49.5± 2.5	140.0± 0.0

[1] Mean deviation of the mean.

In addition to Prospermin KX another batch of Prospermin (KK) was administered to hypophysectomized immature female rats. This material was not assayed in mouse uterine units so that a direct comparison of potency cannot be made. However, because its effects on the ovaries were similar to those obtained with batch KX the results obtained are given here. The extract was administered at 7 different dose levels, the same total doses in milligrams being employed as with batch KX. Groups of 2 to 8 animals were tested and the average ovarian weight was found to increase with the increase in dosage (table 3). Ovarian weight was more markedly increased with this extract than with the other (KX) on 20 and 25 mg. doses but with 30 mg.

TABLE 4. RESPONSES OF HYPOPHYSECTOMIZED IMMATURE FEMALE RATS TO NORMAL MALE URINE EXTRACT

Total dose		No. rats	Mean body weight		Organ weights	
mg.	M.U.U.		Start gm.	End gm.	Ovaries mg. $\pm \Sigma_m$[1]	Uterus mg. $\pm \Sigma_m$[1]
0	0	9	77.1	67.2	6.9±0.3	21.0± 0.5
18	5	3	64.0	56.0	6.8±0.2	28.8± 2.9
36	10	8	67.4	62.2	16.1±3.1	104.6±12.1
72	20	11	69.5	63.5	58.9±5.6	115.0± 7.0
144	40	7	70.7	63.1	72.7±5.0	127.3± 8.3

[1] Mean deviation of the mean.

the effect was less pronounced, possibly because this dose was poorly tolerated, only 2 of the 16 injected animals surviving the 5-day injection period. Ovaries from one animal that had received a total dose of 25 mg. weighed 146 mg. This weight was the greatest of any obtained in the entire series.

The ovaries of the rats after administration of 2.5 or 5 mg. doses contained only a few small vesicular follicles and exhibited fairly general thecal luteinization. Thecal luteinization was predominant with the 10 mg. dose, although a slight increase in the number of small follicles was observed but none were stimulated to medium size. An increased dose (15 mg.) produced the same type ovaries with the exception of a single follicle being stimulated to large size in one ovary. One of the four animals receiving 20 mg. exhibited pronounced follicle stimulation but several of the follicles had thickened granulosa. The ovaries of the other animals on this dose while containing vesicular follicles, some of large size, also had corpora lutea atretica. Higher doses likewise caused the formation of corpora lutea and the stimulation of follicles in the ovaries. Thus this batch (KK) produced the same effect on the ovaries as did batch KX except that with the former the ovarian weight increased proportionally with increase in dose, whereas there was no apparent proportionality between dose and response in the case of the latter.[4]

Normal male urine extract (*L582A* and *L594B*), prepared according to the method of Levin and Tyndale, was administered to a series of hypophysectomized rats. Twenty-three of the 29 rats received *L594B*. Both preparations were of approximately the same potency as assayed by the response of the mouse uterus. Four total doses were tested in groups of 3 to 11 animals. Only 3 rats were tested on the lowest dose (18 mg.) which produced no effect on ovarian weight or histology. However, the three higher doses caused a significant increase in ovarian weight, 144 mg. increasing the average weight 12-fold (table 4).

[4] After completion of this work we were informed by Dr. De Frates of E. R. Squibb & Sons that he believed the preparation Prospermin KX to be slightly contaminated with pregnancy urine gonadotropin. However, preparation KK, not contaminated with PU, produced identical histological changes in the ovaries of hypophysectomized rats.

Ovarian stimulation was limited to the formation of vesicular follicles. Ovaries of large size exhibited many follicles of fairly uniform size with normal granulosa and ova (fig. 5). Corpus luteum formation was not observed in any instance. Furthermore, in the ovaries of only one of the 7 animals that received the largest total dose (144 mg.) was there a suggestion of thecal luteinization.

Any ovarian stimulation induced with this material (*L594B*) was in the nature of follicle stimulation whereas only two of the 82 animals treated with Prospermin exhibited only follicles, all of them, however, showing some thecal luteinization. Furthermore, the ovaries of these two animals averaged 33 mg., whereas the one case of slight thecal luteinization observed after injection of *L594B* occurred in ovaries weighing 86 mg. Although the ovarian weight produced with Prospermin was as great as with *L594B* the ovaries had predominantly corpora lutea with the former and large vesicular follicles with the latter. A total of 144 mg. of *L594B* was necessary to cause an ovarian weight increase comparable to that induced by 30 mg. of Prospermin KX. However, in comparing the two extracts on the basis of mouse uterine units it was found that 144 mg. of *L594B* was equivalent to 40 M.U.U. whereas 30 mg. of Prospermin was equivalent to 3000 M.U.U.

DISCUSSION

Investigations on dogs indicated that Prospermin was capable of stimulating follicles but gave no definite evidence of a luteinizing action (11). Pregnancy urine extract also stimulated the formation of follicles of medium size in the dog without formation of corpora lutea (19). Thus experiments on smaller animals are of more value in determining the qualitative action of a gonadotropin. Although sperm induction was obtained with Prospermin in hypophysectomized ground squirrels and rats, the luteinizing action of this extract was also indicated by the marked enlargement of the accessory sexual organs (13, 14).

An initial indication of a difference in the action of the two types of male urine preparation (Prospermin and the Levin-Tyndale preparation) was observed when these were assayed on mice. Prospermin caused an ovarian weight increase in identical proportion as did pregnancy urine extract. Hemorrhagic follicle formation was observed in the ovaries of more than 50% of the animals receiving 10 M.U.U. of Prospermin, whereas this reaction occurred with a dose of PU between 10 and 20 M.U.U. In contrast, the Levin-Tyndale male urine extract caused more than 100% greater ovarian weight increase than did Prospermin at the same dose level and with no indication of luetinization or blood point formation.

Corpora lutea formation resulted in normal immature female rats with every dose of Prospermin that caused ovarian weight increase. The smaller doses produced corpora lutea in only 1 or 2 animals but with doses that doubled the ovarian weight corpora lutea were observed in every ovary.

Although experiments on normal animals give an indication of the potency and action of extracts, a more justifiable comparison of gonadotropic action can only be made on hypophysectomized animals. These male urine preparations were tested in a manner identical to that used in testing castrate urine (CU), thus permitting a direct comparison of these results (20).

Prospermin was found to be capable of increasing ovarian weight in hypophysectomized immature rats to the same degree as extracts of human menopausal urine (20) and of male urine extract (*L594B*) prepared by the Levin-Tyndale method. However, 3000 M.U.U. of Prospermin were necessary to produce the same average ovarian weight as resulted with 40 M.U.U. of our own preparation. The smaller doses of Prospermin caused excessive thecal luteinization in the ovary, a reaction comparable to that ob-

tained with PU in hypophysectomized rats, and, in addition, both extracts readily produce corpora lutea in normal rats. The larger doses of Prospermin stimulated follicles to large size and caused the formation of corpora lutea. The Levin-Tyndale male urine preparation, however, was devoid of luteinizing action in any of the doses tested.

It therefore appears that the two types of male urine gonadotropin used in these experiments are definitely different in their physiological action. The extracts prepared in this laboratory by tannic acid precipitation and subsequent purification are predominantly follicle stimulating in nature. Although no luteinization was obtained with the doses of this gonadotropin used in our experiments, we feel certain that increasing the dose further would have finally resulted in lutein changes. It has been previously shown (20) that with CU about 100 M.U.U. or about 5 to 10 times the minimal rat ovary stimulating dose is required to produce early lutein changes in the hypophysectomized rat ovary and it is probable that lutein changes would have resulted in the experiments reported here if we had approximately doubled the highest dosage of our preparation.

The other type of male urine preparation (Prospermin) has much more pronounced luteinizing properties and in many respects bears closer resemblance to pregnancy urine gonadotropin than to CU.

The reason for this difference is unknown to us at the present time. Our male urine preparations are much less potent (M.U.U. per mg.) than is Prospermin but that this difference would account for the difference in action between these two preparations and for the similarity between the Prospermin and the PU does not seem likely, particularly in the light of the inability of anyone to change either the potency or physiological effect of urinary gonadotropins by the addition of non-specific substances such as zinc sulphate, and egg albumin. Furthermore, the fact that castrate urine preparations, much more potent in terms of M.U.U. per mg. total solids, whether prepared by our method or any other, behave almost exactly as do the male urine extracts made by us affords additional evidence that discrepancies noted here are not due to differences in degree of contamination with non-specific substances.[5]

SUMMARY

A study has been made of the gonadotropic action of the urine of normal men using the Squibb product Prospermin as well as concentrates prepared by tannic acid precipitation and subsequent partial purification.

The two types of preparation are strikingly dissimilar in effect, particularly as indicated by histological changes in the ovary of the hypophysectomized rat. Prospermin has very pronounced luteinizing properties, in this respect resembling the gonadotropin of human pregnancy urine. However, Prospermin is also able to stimulate follicular development in the hypophysectomized rat ovary. Our own preparations are effective follicle stimulators but are entirely devoid of luteinizing ability in the doses tested. Our extracts closely resemble the urinary gonadotropin of ovariectomized and postmenopausal women. It is believed that if larger doses were used lutein changes would be obtained.

[5] During a discussion of these results it was suggested by Drs. Morrell and De Frates that the difference in methods of preparation might be responsible for the differences in physiological action noted above. We therefore made an extract of normal male urine using the method furnished us by Dr. De Frates. The final powder was found, when assayed by the mouse uterus technic, to have an activity of the same order of magnitude as that of extracts prepared by our own method. Unfortunately, only a small amount of material was obtained and therefore only a few tests of its action in hypophysectomized rats could be made. In these tests, however, even as much as 40 M.U.U. of this preparation produced little or no ovarian stimulation. This appears to agree with the results obtained with the Prospermin. It should be added, however, that the material prepared by us by the Squibb method was very poorly tolerated by the test animals and this factor must also be considered in any interpretation of the results.

These properties of the two preparations are confirmed by the results of the studies in normal immature rats and mice.

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"MAMMOGEN" AND THE TREATMENT OF SPAYED HYPOPHYSECTOMIZED RATS WITH LIPOID EXTRACTS OF CATTLE PITUITARY

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THE INTRODUCTION of the use of hypophysectomized animals for the study of mammary gland development (1–4) soon made it clear that the interpretation of the earlier results obtained by the use of ovarian hormones in stimulating these glands would have to be revised. Selye and Collip (4) concluded that "Ovarian hormones act on the mammary gland indirectly by way of their pituitary-stimulating effect" on the basis that estrone always produced mammary stimulation in intact female rats but failed to do so in the absence of the pituitary or when the pituitary was replaced with crude extracts containing at least adrenotrophic and gonadotrophic activity. Related experiments based on hypophysectomized rats, mice, rabbits, guinea pigs and ground squirrels have been largely confirmatory (for reviews see Turner (5) Riddle (6)).

The inference that the anterior pituitary may exert a direct mammotrophic action received little support in first reports describing the use of pituitary extracts (4, 8, 10) and implants (7, 8) in hypophysectomized recipients. Gomez, Turner and Reece (9) implanted the pituitaries of rats which had been receiving estrogen into normal or gonadectomized hypophysectomized male and female (10) guinea pigs and obtained definite duct growth and lobule-alveolar development. For this substance they have used the apt term, pituitary mammogen (11). Reece and Leonard (12) obtained confirmatory duct growth in gonadectomized hypophysectomized female rats receiving implants of rat pituitaries; the mammogen content of the donor pituitaries did not appear to be either conditioned or altered by estrogen treatment.

Lewis and Turner (13) noted a decrease in the mammogen content of the pituitaries of pregnant cattle following desiccation with acetone. Investigation of the extracted lipoid material revealed that it possessed a duct growth factor referred to as mammogen I (14). Their assays of this material were carried out on normal or gonadectomized male or female mice. The present report concerns our inability to confirm the lipoid-like nature of mammogen, and substantiates the claim that the pituitary stimulates mammary development directly.

EXTRACTION PROCEDURE

Lewis and Turner (13) have claimed that 90% of the mammogenic potency of the anterior pituitary from pregnant cattle was extracted with 86% alcohol. However, their best results were obtained by extraction of the fresh tissue with several volumes of an ether-alcohol mixture (1:3) at 50° C. By this method it was claimed that 100% of the potency was to be found in the ether-alcohol residue. In the present work this

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procedure was followed, except that the ground pituitary tissue was thoroughly dried at room temperature so that the lipoids could be extracted more efficiently.

Fresh frozen female cattle pituitaries were ground and dried in vacuum desiccators over sulfuric acid and potassium hydroxide. The dry weight was about 20% of the weight of the frozen glands. The dried material was further ground in a ball mill and put through a 100 mesh sieve. This powder was assayed for mammogen content.

One hundred grams of the dry powder was placed in a round bottom flask fitted with a stirrer and condenser, one liter of ether-alcohol mixture (1:3) was added, and the flask was heated to 40°–50° C. for 6 hours with constant stirring. The solvents were decanted and the process repeated with two more portions of the ether-alcohol mixture. The combined solvents were filtered through a layer of supercell to remove finely divided protein material, and the liquids removed in vacuo under 50° C. The lipoid residue amounted to 10% of the dry weight, or about 2% of the wet weight of the pituitaries. This residue was assayed for mammogen content. The material insoluble in ether-alcohol was dried and also assayed.

The possibility remained that preliminary drying of the pituitary glands before extraction in some way rendered mammogen unextractable with ether-alcohol mixtures. Fresh frozen female cattle pituitaries were ground and extracted directly without preliminary drying according to the procedure used with the dried glands. In this case a somewhat larger amount of ether-alcohol residue was obtained, probably because of the considerable amount of water present in the initial portion of ether-alcohol used for extraction.

Assay

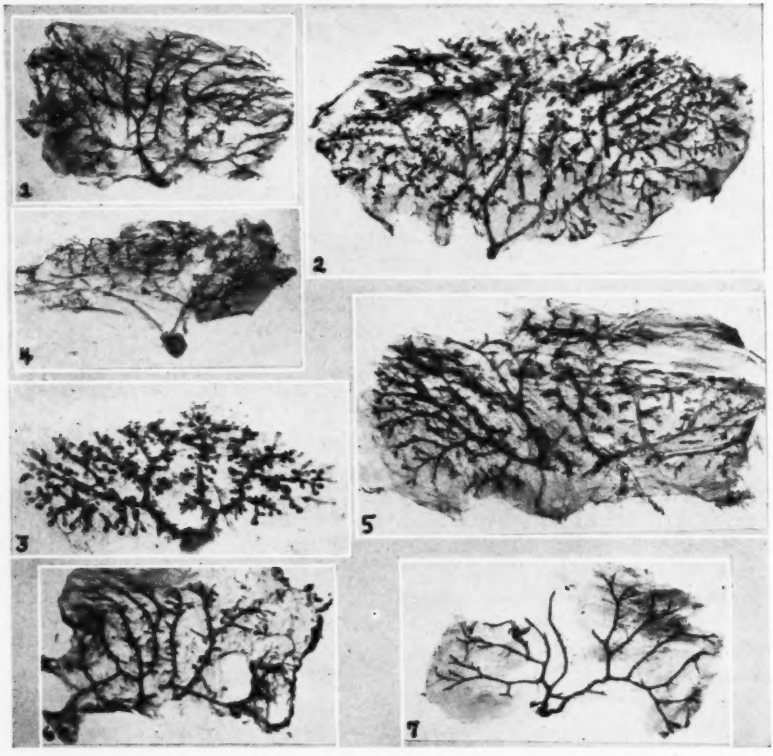
The assays were made on female rats which were spayed and hypophysectomized at 21 days of age. Subcutaneous injections were started one week later and continued for 6 days. At necropsy on the day after the last injection, a mammary spread was prepared from the left abdominal gland. It was found essential to study a selected gland in order to avoid being misled by the difference in size that is normally present in the glands of an individual animal. The variation even between such topographically comparable glands in a group of 10 control animals was also sufficient to make determinations of minimum stimulation difficult. Estimations of the degree of response were extremely rough and were based upon either obvious extension of the gland over a greater area with increased thickness of the ducts or on an associated increase in number and size of the end-buds or both.

RESULTS

The desiccated and powdered pituitaries, injected as a suspension in saline, produced unquestionable mammary growth. Groups of 5 to 7 animals each received dosages of 25, 50, 100 and 400 mg. E.F.G. (Equivalent of Fresh Gland). The response to 25 mg. was slight but appeared to be definite. Unquestionable extension of the duct system was produced by 50 mg. and the greatest extension observed was produced by 100 mg. (fig. 1, 2). Marked development of the end-buds with reduced duct extension characterized the response to 400 mg. E.F.G. (fig. 3). Administration of greater doses did not seem feasible because of untoward local reactions to the large amount of suspended material.

The waxy lipoid fraction extracted by the ether-alcohol mixture was dissolved in pure olive oil and injected in doses of 1.7 and 6.8 mg. representing 100 and 400 mg. E.F.G. respectively. Examination of the mammary glands of these animals did not reveal that any stimulation had been induced (fig. 4). A more critical assay of this material was carried out on a group of 5 animals which had been spayed and hypo-

physectomized one month before beginning treatment. A total dose of 54 mg. of the lipoid material (or 3200 mg. E.F.G.) was given over 8 days. A similar group of 5 untreated animals served as controls. It was not possible to distinguish the extremely atrophic duct system of the control glands (fig. 6) from those of animals receiving this large dose (fig. 7). In other experiments it was found that the powdered whole glands would produce mammary growth in rats which had been hypophysectomized for 2



LEFT ABDOMINAL MAMMARY GLANDS OF SPAYED HYPOPHYSECTOMIZED IMMATURE FEMALE RATS (×4.7).

Fig. 1. Control, operated 14 days. Fig. 2. Received original powdered cattle pituitary, 100 mg. E.F.G. (Equivalent of Fresh Gland) in 6 days beginning one week after operations. Fig. 3. 400 mg. E.F.G. original powdered gland. Fig. 4. Ether-alcohol extract, 6.8 mg. lipoid material or 400 mg. E.F.G. total dose. Fig. 5. Ether-alcohol insoluble (glandular) residue 200 mg. E.F.G. (Ages, postoperative intervals and time of treatment same for fig. 3, 4, 5 as for fig. 2 above). Fig. 6. Control, operated 39 days. Fig. 7. Treated with 54 mg. lipoid extract (or 3200 mg. E.F.G.) over 8 days beginning 30 days after operations.

months. The ether-alcohol extracts prepared from fresh (undried) glands likewise did not exhibit any mammary stimulating activity (largest dose = 46.8 mg. extract or 1200 mg. E.F.G.).

It was of interest to examine whether the mammogenic potency had been removed from the pituitaries by the ether-alcohol extraction. Groups of 5 animals each were injected with 50 and 200 mg. (E.F.G.) of the tissue residue. The lesser dose produced

a minimum effect but with 200 mg. marked duct growth was observed (fig. 5). It was evident that there had been some loss of potency.

Body growth was produced by the original powdered gland and by the tissue residue after ether-alcohol extraction but not by the lipoid extract. The groups receiving 25 and 50 mg. E.F.G. of the original pituitary powder showed average gains per individual of 14.1 and 15.8 gm. respectively. Fifty and 200 mg. E.F.G. of the tissue residue after extraction each produced average gains of 14.4 gm. The animals receiving the ether-alcohol extract at a dosage of 3200 mg. E.F.G. exhibited an average gain of 2.9 gm. and their controls 3.4 gm.

DISCUSSION

The recent claims (13–16) that ether-alcohol extracts of cattle pituitary will produce mammary duct growth appear to be untenable. We have not been able to demonstrate that such extracts are active but we have employed a different test than was used by the Missouri workers. The establishment of the existence of the lipoid-soluble mammogenic factor must rest on its behavior as a substitute for anterior pituitary tissue administered to hypophysectomized gonadectomized animals. Our source material possessed unquestionable mammogenic activity, no trace of which could be found in the extracted lipoid fractions. The fact that some loss of activity occurred in the original material during this extraction appears to be due to destruction of the active substance rather than to extraction.

The evidence at hand permits no more than speculation on the interesting problem of whether the pituitary mammogenic activity is a part of, or distinct from, the pituitary growth complex. In our data mammary stimulation and body growth were associated but not always correlated as to amount. Some experimental evidence that these activities may not be identical can be adduced from the data of Reece and Leonard (12) in that mammary stimulation but no body growth followed the implanting of pituitaries from estrogen treated rats into hypophysectomized recipients. Also, Nathansen, et al. (17) obtained better mammary growth in hypophysectomized rats with growth hormone plus estrogen than with growth hormone alone. Pertinent also is the observation that the mammary glands of rats gonadectomized prior to puberty never developed beyond the infantile condition whereas body growth continued unrestricted (18). It is obvious that the final solution of this problem must await the isolation of the pituitary growth factor(s) or 'pituitary mammogen.'

SUMMARY

Female cattle pituitaries desiccated in vacuo, powdered, and injected as a suspension in saline induced definite mammary duct growth and end-bud formation in spayed hypophysectomized immature female rats. A lipoid fraction extracted from this pituitary powder or from fresh undried pituitary glands with an ether-alcohol (1 : 3) mixture produced none of the signs of mammary stimulation. The mammogenic activity of the tissue residue was somewhat reduced by the extraction procedure but this loss of activity was not accounted for in the lipoid fraction. Body growth was produced by the pituitary tissue, before and after extraction, but not by the lipoid fraction. The amount of body growth and the extent of mammary stimulation were not always correlated.

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EFFECT OF METHOD OF DESICCATION AND STORAGE ON THE GONADOTROPIC ACTIVITY OF THE PITUITARY GLAND[1]

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PITUITARY TISSUE that is to be kept for various periods of time may be stored in the frozen or desiccated condition. During the period of storage of the tissue in the frozen condition, or as a result of the method of desiccation, there may be a loss of biological activity when compared with fresh tissue. There are not, however, many reports concerned with the effect of storage or desiccation on the gonadotropic activity of the pituitary gland. Smith and Engle (1) reported in 1927 that implants of beef pituitary glands 8 hours old were not as effective as those 1 hour old. Wallen-Lawrence and Van Dyke (2) showed that after storing the pituitary glands of rats in the frozen condition for 9 to 36 days the gonadotropic activity was decreased when compared with that of fresh glands. Fraenkel-Conrat, Simpson and Evans (3) reported recently that a loss of gonadotropic and adrenotropic activity follows repeated freezing and thawing or grinding of fresh sheep pituitary glands when compared with untreated fresh glands. No comparison was made, however, between the effect of desiccation and that of freezing. Hill (4) recorded that acetone-dried beef pituitary powder was as effective in causing ovulation in the rabbit as that prepared by extracting the gland with dilute alkali.

A study of the reports just reviewed made it seem of importance to investigate the effect of desiccation with alcohol, acetone or air on the gonadotropic activity of the pituitary gland. A comparison of the influence of these agents with that of storing fresh glands in the frozen condition was also considered to be of value.

PROCEDURE

Whole fresh pituitary glands of rats, sheep, and chickens were collected and frozen[2] and those from each species were thoroughly triturated and divided into 5 or 6 equal portions. Each of the individual portions was subjected to one of the following procedures.

(*a*) *Desiccation by acetone.* The material was thoroughly dehydrated by placing the triturated glands in 10 or 15 volumes of acetone for 4 to 5 hours, followed by centrifugation and decantation. The process was repeated 4 times.

(*b*) *Desiccation by alcohol.* The triturated glands were dehydrated by treating them with 10 to 15 volumes of 95% alcohol for 4 to 5 hours. After centrifugation and decantation the process was repeated 4 times.

(*c*) *Desiccation by evaporation.* In this process the fresh glands were crushed between two glass plates. Enough pressure was applied to completely flatten the tissue into a moist film. The glass plates were then pulled apart and placed in a drier where warm air of approximately 36° C. was forced over the tissue by an electric fan. This procedure required from 3 to 10 minutes depending upon the amount of tissue being

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[2] The fresh-frozen pituitary glands will be referred to as either fresh or fresh-frozen.

dried. The dried material was scraped off the plates with a sharp single-edged razor blade and then ground to a fine powder.

(*d*) The remaining aliquots of fresh frozen tissue were not desiccated. One aliquot was assayed immediately with acetone, alcohol and air-dried powders and the others were stored at −10° C. for 35 or 80 days before injection.

The fresh-frozen material was prepared for assay by thoroughly triturating the tissue in a centrifuge tube with a glass rod which had been ground to fit the bottom of the tube. Water was added and the mixture flushed through hypodermic needles of a progressively smaller bore until a fine suspension was obtained. The acetone, alcohol and air-dried sheep and beef pituitary glands were pulverized with a mortar and pestle and the powder obtained was sifted through a fine screen with openings of 0.0087 inches. It was not necessary to follow this procedure in the treatment of the pituitary glands of the other species since they did not have as much connective tissue. The powder was further pulverized and then prepared for injection by suspending it in water.

The assays were made on 21-day-old female rats which were injected with 0.5 cc. twice daily for 4.5 days and killed on the morning of the sixth day. The volume injected was the same for both the desiccated and frozen material. All of the injections of dried glands were made on the basis of fresh-frozen equivalents. This procedure made it possible to compare directly the effect produced by desiccation with that obtained with fresh-frozen glands. Thus each of the rats injected with sheep pituitaries received 200 mg. equivalent of fresh gland. Each of those given chicken pituitaries was injected with 150 mg. equivalent of fresh tissue, while the rat pituitaries were administered in doses of 16 mg. equivalent of fresh-frozen tissue. The experiments with rat and sheep pituitaries were repeated a second time and the results are recorded in table 1 as those of Series I and II.

TABLE 1. THE EFFECT OF METHODS OF DESICCATION AND STORAGE ON THE GONADOTROPIC ACTIVITY OF PITUITARY GLANDS

Treatment	Rat pituitary Series I		Rat pituitary Series II		Chicken pituitary		Sheep pituitary Series I		Sheep pituitary Series II		Beef pituitary	
	16 mg.[1]		16 mg.[1]		150 mg.[1]		200 mg.[1]		200 mg.[1]		500 mg.[1]	
	No. rats	Ov. wt.±SE	No. rats	Ov. wt.±SE	No. rats	Ov. wt.±SE	No. rats	Ov. wt.±SE	No. rats	Ov. wt.±SE	No. rats	Ov. wt.±SE
Air-dried	7	73± 6.76	6	88 ± 4.49	8	28.6±3.04	8	116 ± 8.68	—	—	6	60±3.42
Acetone-dried	7	27± 2.4	6	57 ± 5.78	8	18.5±1.28	8	62 ± 6.59	6	36±4.99	6	32±4.85
Alcohol-dried	—	—	6	66 ± 5.57	8	19 ±1.21	8	62 ± 4.6	—	—	—	—
Fresh-frozen	7	100± 9.66	6	126 ±17.6	8	36 ±6.64	8	112 ±10.32	6	85±5.05	—	—
Frozen for 35 days	7	95±15.37	6	88.5±12.34	8	30 ±2.45	8	110.5± 5.24	—	—	—	—
Frozen for 80 days	7	67± 8.76	6	66 ± 5.53	8	23 ±1.97	8	102 ± 8.49	—	—	—	—

[1] Equivalent of fresh tissue.

RESULTS

The data presented in table 1 indicate that desiccation with alcohol and acetone is followed by a considerable loss of gonadotropic activity when compared with the response obtained by the injection of equivalent doses of fresh pituitary tissue. The average ovarian weight obtained with the fresh glands of the rat (Series I and II), sheep (Series I and II) and chicken were 100, 126, 112, 85 and 36 respectively, as compared to the average ovarian weights of 27, 57, 62, 36 and 18.5 respectively, when the tissue was acetone-dried. The loss of activity following alcohol desiccation was approximately the same as that when acetone was used. Drying with air, however,

resulted in little or no significant loss of potency as compared with fresh material. Injection of an equivalent dose of fresh material in the air-dried form gave average ovarian weights of 73 and 88 mg., respectively, for the two series receiving rat pituitary, 28.6 mg. for those receiving chicken, and 116 mg. for those receiving sheep pituitary. Comparison of these ovarian weights with those produced by injecting fresh glands demonstrated that there was no difference which was statistically significant.[3] In the case of the first series of injections of rat pituitary it was found that the difference between the average ovarian weights obtained with the air-dried and fresh material was statistically significant. In addition to the above experiments, beef pituitary glands were subjected to the acetone and air-drying methods of desiccation to determine their effect on the gonadotropic potency. These results are similar to those given for sheep, rat and chicken hypophyses (table 1). The ovarian response with air-dried material was twice that obtained with acetone powder. An analysis of the results shows that with all species of glands studied desiccation with alcohol or acetone caused a significant decrease in activity when compared with fresh glands, whereas air-drying resulted in little or no loss.

TABLE 2. THE EFFECT OF ACETONE RESIDUE WHEN INJECTED ALONE OR IN COMBINATION WITH THE ACETONE-DESICCATED SHEEP PITUITARY POWDER

Dose	Fresh pituitary		Acetone-dried		Acetone residue		Acetone residue + Acetone-dried	
	No. rats	Ovarian wt.+SE	No. rats	Ovarian wt.+SE	No. rats	Ovarian wt.+SE	No. rats	Ovarian wt.+SE
200 mg.[1]	6	85±5.1	6	36±4.99	5	13±0.64	6	39±9.95

[1] Equivalent of fresh tissue.

There was no significant loss in gonadotropic potency following storage of the glands at −10° C. for 35 days, but storage of the material for 80 days at the same temperature resulted in a definite loss of activity in the rat and chicken glands but no significant loss of activity in the sheep glands.

The activity lost by desiccating sheep pituitary glands with alcohol or acetone was not present in the alcohol or acetone as the residues obtained on evaporation of these liquids at 36° C. were not active when given in equivalents of 1750 mg. of fresh pituitary tissue. This finding is like that reported by Wallen-Lawrence and Van Dyke (2) for rat glands. For the purpose of determining whether there was any substance present in the acetone residue which might be synergistic with the powder obtained from desiccation of the pituitary glands with this solvent, the residue resulting from the evaporation of the acetone was suspended in water and injected alone and in combination with the acetone-dried powder. Two hundred milligrams equivalent of fresh tissue was administered in all cases. The data presented in table 2, show no evidence of synergism since there was no difference between the average ovarian weight obtained with the acetone-dried powder and the acetone-dried powder plus the acetone residue.

DISCUSSION

The results of this investigation show that air-drying is decidedly more advantageous than the other methods of desiccation in preserving the gonadotropic activity of the fresh frozen gland. Not only is there little or no loss of activity as compared

[3] The measure of the significance of a difference between means was determined by the method described by Davenport and Ekas (5). This consisted essentially of obtaining the quotient of the mean difference divided by the standard error of the mean difference. The resulting value, designated as t, is referred to Table XIII of t values to ascertain whether the difference between the means was significant.

with the fresh gland but the powder resulting from this method formed a more stable suspension than the powder derived from acetone or alcohol desiccation.

From the standpoint of immediate assay of frozen pituitary tissue suspension of the fresh glands gave the best results for rat pituitary, but with the sheep and chicken hypophysis the gonadotropic potency of the powder obtained from air-drying was not significantly less than that of the fresh gland. When considering the question of storage of the air-dried powder, table 3 shows the results of assaying rat pituitary stored

TABLE 3. EFFECT OF THE LENGTH OF STORAGE ON THE GONADOTROPIC ACTIVITY OF AIR-DRIED RAT PITUITARY POWDER

Dose (dry powder)	Before storage		Stored 3 months		Stored 1 year	
	No. rats	Ovarian wt.±SE	No. rats	Ovarian wt.±SE	No. rats	Ovarian wt.±SE
2 mg.	3	64±10.18	7	64±7.77	6	58±11.87

for various periods of time. The material assayed 3 months after the original assay had been made did not show any decrease in potency and after an interval of one year there was no demonstrable loss of activity. Storage of the fresh pituitary for 80 days caused a definite and highly significant decrease in gonadotropic activity of the rat and chicken pituitaries but not of sheep glands. It is conceivable that this difference may be the result of a species difference in the quantity of autolytic enzymes present.

The loss of activity following alcohol or acetone treatment cannot be explained on the basis that some active material was discarded with the dehydrating agent since evaporation of these agents and assaying of the remaining residues failed to reveal the presence of gonadotropic activity. Likewise, it cannot be explained on the basis of removal of a synergist by the acetone since recombination of the inactive acetone residue with the acetone-dried powder did not show an increase in the average ovarian weight over that obtained with the dry powder alone. The loss of gonadotropic activity following acetone desiccation, however, may be the result of either inactivation of the hormone, denaturization of the gland proteins, disturbance of the protein-lipoid relationship, or a combination of these factors.

SUMMARY

The results of a study of acetone, alcohol and air-drying methods of desiccation on the gonadotropic activity of rat, sheep, chicken and beef pituitary are reported. Desiccation by the air-drying method entailed little or no loss of activity while acetone and alcohol desiccation resulted in at least a 50% decrease in potency as compared to that of fresh glands. Injection of alcohol and acetone residues indicated that the loss of activity by the use of these solvents cannot be explained on the basis of activity extracted with the solvents. Storage of fresh frozen glands for 35 days caused no significant loss of gonadotropic potency, but storage for 80 days resulted in a significant loss of activity in the glands of rats and chickens but not in those of sheep.

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GONADOTROPIC STIMULATION IN INTACT AND THYROIDECTOMIZED ADULT FEMALE RATS

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STUDIES on the influence of delayed resorption of gonadotropic hormones upon ovarian hypertrophy have been confined to immature test animals both normal and hypophysectomized and with good reason, since the complication arising from the presence of endogenous hormone or a somewhat stimulated ovary is negligible.

In the present paper, the problem has been extended to the adult rat in order to include the factors of endogenous hormone and developed ovary. In earlier studies (1), we concluded that thyroidectomy produced results similar to delayed resorption, and thyroxin administration produces results similar to rapid resorption on the basis of the decrease or increase in rate of exchange of body fluids which these procedures bring about, so decreasing or increasing the rate of hormone liberation. It was considered desirable also to test out this hypothesis in the adult rat, which had been subjected to early thyroidectomy, since the influence of the thyroid upon the ultimate development and response of the ovary was not known.

EXPERIMENTAL

In each experiment, groups of 6 or 7 animals were used and a comparison was made between litter mates. Thyroidectomy was performed at the age of 22 to 23 days by the technic previously described (1). Dosage of the gonadotropic hormone was begun 41 days later, and was made once daily for 4 days. The animals were killed the 5th day after the initial dosage. The pituitary gonadotropic hormone was the same stable powder used in former experiments (1, 2), so that data obtained for the adult rat may be compared with data obtained for the immature rat. When copper was used to delay resorption, it was incorporated in the pituitary hormone solution according to the specific directions previously given (3).

In this study was added another objective measure to gauge degree of thyroidectomy. Blood pressure readings were made by the method of Williams, Harrison and Grollman (4). In normal rats the blood pressure readings by this method ranged between 110 and 130 mm. In thyroidectomized rats the range was found to be 80 to 98 mm.

RESULTS

Since there are no data available in the literature for the effect of thyroidectomy after a 40-day period on 23-day-old rats, we have included (see table) data on the thymus, adrenals and pituitary, in addition to the results on body, heart and ovarian weights which served as the objective measures in the present experiment. Per 100 gm. body weight the ovaries, thymus, heart and adrenals are smaller in thyroidectomized rats, but the differences on this basis are not striking. The pituitary glands on the other hand increase 100% in weight. These results are in substantial agreement with those of Hammett (5) obtained on 150-day-old rats operated at 100 days of age. There is a marked lag in body weight increase in the thyroidectomized rats. Occasionally a thyroidectomized rat was found which gained in body weight at a rate comparable to the control and in which thyroid tissue was not found on section

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of autopsy material. These rats and their litter mates were not used in the experiments.

There are available data for 4 groups of normal rats, 6 rats in a group, killed at 68 days of age. The average ovarian weights for these groups are 54 ± 4, 43 ± 6, 47 ± 4 and 53 ± 4. The dosage of 1 mg. Cu as $Cu(OH)_2$, experiment 5, produced no significant change in ovarian weight, 48 ± 4 versus 53 ± 4 (litter-mate controls). The dosage of 1 mg. or 2 mg. gonadotropic hormone in soluble form, in experiments 4 and 1, produced no significant increase in ovarian weight. In experiment 1, the figures are 51 ± 5 versus 54 ± 4 (litter-mate controls). In experiment 4, there are no controls. The figure 61 ± 5 is not significantly different, though it probably indicates a slight effect. The dosage of 2 mg. gonadotropic hormone with 1 mg. Cu in experiment 2 produced a highly significant increase in ovarian weight, 85 ± 9 versus 43 ± 6 (litter-mate controls).

There are available data for 3 groups of untreated thyroidectomized rats, 6 rats in a group, killed at 68 days of age. The average ovarian weights for these groups are 22 ± 6, 23 ± 3 and 25 ± 4. Dosage of 1 mg. and 2 mg. of soluble gonadotropic hormones produced significant increases in ovarian weights in the thyroidectomized rats, 44 ± 5 versus 22 ± 6 (litter-mate controls) in experiment 1. In experiment 4, there were no litter-mate controls but the ovarian weight of 53 ± 3 is significantly greater than the control data of the other series. Two mg. of gonadotropic hormone administered as the insoluble copper combination produced a significant increase in the ovarian weight of the thyroidectomized rats in experiment 2.

DISCUSSION

Control for copper alone. We previously found that in the immature rat (2) copper administered at a site removed from the injection site of the gonadotropic hormone produced no augmentation in ovarian weight. Evans, Hines, Ceithaml and Koch (6) showed that the injection of copper alone to the immature rat produced no increase in ovarian weight. Fevold et al. (7) demonstrated that the intravenous injection of copper salts into mature rabbits caused ovulation and that copper salts produced no augmentation of the gonadotropic effect in hypophysectomized immature rats. They concluded that the activity of copper salts apparently could not be explained on the basis of decreasing the rate of absorption but might be due to a catalytic action in the synergistic interaction of FSH and L H. Brooks et al. (8) showed that transection of the hypophysial stack abolished ovulation following intravenous injection of copper in the rabbit, and concluded that this specific phenomenon is due to a nervous mechanism as we have previously suggested (2).

In the present work (experiment 5), we injected the copper in mature rats whose endogenous supply of gonadotropic hormone was established. The failure to influence the ovarian weight indicates that copper given subcutaneously in the rat neither stimulates the liberation of endogenous hormones (nervous mechanism) nor does it produce any synergism between the endogenous hormones.

The intact adult rat. These experiments indicate that in the adult rat the factor of delayed resorption is of the same order as it is in the immature rat. The experiments also demonstrate that under conditions assuring maximum response to the gonadotropic hormone, the magnitude of response (on an absolute basis) is roughly proportional to the dosage on a weight basis. Thus, in the 50 gm. normal immature rat 1 mg. hormone per 100 gm. body weight produced a 32 mg. increase in ovarian weight; 2 mg. per 100 gm. body weight produced an 80 mg. increase in ovarian weight. As the present experiment shows in the 130 gm. adult rat, 1.5 mg. hormone per 100 gm. body weight produced a 42 mg. increase in ovarian weight.

TABLE 1. INFLUENCE OF DELAYED RESORPTION AND OF THYROIDECTOMY ON GONADOTROPIC STIMULATION IN MATURE RATS

Experiment	State of rats	Dose per rat	Mean body and organ weights						Mean weight per 100 gm. body weight				
			Body	Heart	Thymus	Adrenal	Pituitary	Ovaries	Heart	Thymus	Adrenal	Pituitary	Ovaries
		mg.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
1	Normal		150±7	621±30	430±24	52±2	7.1±0.4	54±4	0.41±0.01	0.29±0.01	35±2	4.7±0.2	36±2
	Normal	2	154±5	627±14	447±28	48±4	6.1±0.2	51±5	0.41±0.01	0.29±0.005	31±2	4.0±0.1	33±3
	Thyroidectomized		84±7	297±15	194±19	26±2	7.6±0.5	22±6	0.36±0.02	0.23±0.01	31±3	9.2±0.6	24±5
	Thyroidectomized	2	95±9	305±23	192±28	26±3	7.7±0.4	44±5	0.33±0.01	0.20±0.01	28±1	8.5±0.8	47±4
2	Normal		143±5	586±28	384±23	50±5	6.5±0.3	43±6	0.41±0.01	0.27±0.02	35±3	4.0±0.4	31±4
	Normal	2+1 mg. Cu	130±6	556±29	312±14	48±4	7.3±0.6	85±9	0.42±0.01	0.24±0.01	37±3	5.6±0.3	65±7
	Thyroidectomized		82±4	282±13	199±15	22±2	7.1±0.5	22±3	0.34±0.02	0.24±0.04	27±1	8.6±0.5	26±3
	Thyroidectomized	2+1 mg. Cu	78±2	300± 7	110± 8	26±1	8.3±0.5	65±4	0.39±0.01	0.14±0.01	34±1	10.7±0.7	84±4
3	Normal		153±5	588±27	426±15	43±3	5.8±0.5	47±4	0.39±0.01	0.28±0.01	28±1	3.8±0.3	31±2
	Thyroidectomized		93±5	327±17	181±13	24±1	7.4±0.6	25±5	0.35±0.01	0.19±0.01	26±1	8.2±0.9	26±5
4	Normal	1	160±7	635±28	450±34			61±5	0.40±0.02	0.28±0.02			38±2
	Thyroidectomized	1	126±4	428±36	303±12			53±3	0.34±0.01	0.25±0.01			43±2
5	Normal		146±6	587±32	446±21	52±3		53±4	0.41±0.01	0.31±0.02	36±1		36±2
	Normal	1 mg. Cu	138±6	586±33	354±35	50±2		48±4	0.43±0.01	0.26±0.02	36±1		35±3

The adult thyroidectomized rat. In these experiments for the adult rat as in those previously reported for the immature rat, the dose of gonadotropic hormone which when given in soluble form is without appreciable effect in the normal rat, produces a pronounced ovarian weight augmentation in thyroidectomized rats. The factor of body weight enters into the adult rat experiment because during the 40-day period following thyroidectomy the controls gain about 60 gm. in body weight over the operated rats. This factor is, however, controlled in our experiments because the dosage of hormone was given at two levels. Thus, in experiment 4 the thyroidectomized rats received 0.8 mg. hormone per 100 gm. body weight. In experiment 1, the normal rats received 1.3 mg. hormone. The latter dose produced no effect, the former considerable augmentation. The augmentation produced by the soluble compound in thyroidectomized rats is less than that produced by the same amount of compound given as the insoluble copper combination. The ovarian weight increase produced by the insoluble compound in thyroidectomized rats is the same as that produced by the insoluble compound in normal rats if dosage per rat is the same. The increase in ovarian weight per body weight is proportional to dosage per body weight. Thus when prolonged delayed resorption and hence maximum gonadotropic response for a given amount of hormone is obtained in normal rats, the added delay in resorption brought about by thyroidectomy is without further influence.

SUMMARY

Copper administered subcutaneously to the adult rat produces no effect upon the ovarian weight; viz., produces no nervous stimulation leading to endogenous hormone liberation, nor synergism between endogenous hormones.

The response of the ovary of the young or old intact rat to pituitary gonadotropic hormone, administered so as to produce its maximum effect for a given dose, is roughly proportional to dose per body weight; viz., under conditions assuring maximum response, the adult ovary has not become refractive to exogenous hormone.

Forty-one days after thyroidectomy the ovary of the adult rat is responsive to exogenous pituitary gonadotropic hormone stimulation.

The gonadotropic response of the adult (68-day-old) female rat, thyroidectomized at the age of 21 days, is strictly analogous to the response of the immature rat thyroidectomized at the same age and dosed 2 to 3 days later. The dose of pituitary gonadotropic hormone which when given in soluble form is without appreciable effect in the normal rat, produces a pronounced ovarian weight augmentation in the thyroidectomized rat. However, under conditions (dosage of insoluble copper combination) eliciting maximum response for a given amount of pituitary hormone in the intact rat, thyroidectomy is without further influence.

It is concluded that when thyroidectomy exerts an augmentation effect to gonadotropic hormone administration, the phenomenon may be explained by the decrease in rate of exchange of the body fluids, which this procedure brings about, so decreasing the rate of hormone resorption.

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EFFECT OF INCREASING THE NUMBER OF DAILY INJECTIONS OF GONADOTROPIC PREPARATIONS ON THE OVARIES OF IMMATURE RATS[1]

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A NUMBER OF DIFFERENT SUBSTANCES which have no gonad stimulating activity when administered alone have been found to increase the effectiveness of certain pituitary gonadotropic extracts when they are mixed in vitro with the extracts and injected into immature female rats. These augmenters include extracts of normal and castrate human urine, normal and pregnant blood sera (1–5), extracts of milk, beef liver and thyroid (5), extracts of yeast (6), casein and egg albumin (7), leucocytes (8), zinc and copper salts (6, 9–11), formed elements of blood (12), heme and hemoglobin (13), and chlorophyll (14).

The effectiveness of pituitary gonadotropic extracts may be increased also by making more frequent injections as has been shown by Bischoff (15) and Maxwell (9) who reported that pituitary gonadotropic extracts are more effective when they are administered 5 or 6 times a day as compared with once daily. This method of administration, however, did not produce as great an increase as was obtained with some augmenters, notably heme. It seemed of importance, therefore, to determine how much more effective certain of the gonadotropic substances would be if the number of injections was increased from 2 to 24 per day, and to compare the results obtained with those produced by heme-gonadotropic extracts.

EXPERIMENTAL

The following pituitary gonadotropic preparations were used in this study: (*a*) an aqueous extract of sheep pituitary powder which had inert protein removed by treatment with globin; (*b*) the same extract to which was added 4 mg. of heme for each 50 mg. equivalent of dried sheep pituitary powder (16); (*c*) an aqueous extract of beef pituitary powder; (d) an aqueous extract of horse pituitary powder and (*e*) two follicle stimulating extracts, one of which was prepared by a slight modification of the method of Fevold (17), and the other by the trypsin method (18). In addition to these gonadotropic preparations, use was made of pregnant mare serum in both the untreated and purified forms and an extract of human pregnancy urine prepared by precipitating the active substance with ethyl alcohol. The pituitary preparations were administered on the basis of milligrams equivalent of dried gland whereas the pregnant mare serum and human pregnancy urine were given in terms of cubic millimeters or cubic millimeters equivalent respectively.

Female rats of the Sprague-Dawley strain which were 21 days old at the beginning of the injection period were used for assaying the preparations. The hypophysectomized rats had their pituitaries removed when 21 days old and received their first

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injections when 22 days of age. Those rats receiving the hourly injections were given 0.05 cc. of the gonadotropic preparations each hour for 114 hours. One series of rats was injected with 0.10 cc. of human pregnancy urine preparation every 2 hours for 114 hours. Other rats of the same age were injected with 0.60 cc. of the gonadotropic preparations on the evening of the first day and twice daily with this amount for 4 days. All rats were killed on the morning of the sixth day at which time the ovaries were removed, weighed and examined for the relative numbers of follicles and corpora lutea.

RESULTS AND DISCUSSION

The data given in table 1 show that the rats which received hourly injections of the sheep pituitary extract had heavier ovaries than those given the same quantity in 2 injections per day. The ovarian weights obtained depended upon the amount of the extract given and the number of injections made. Thus an average ovarian weight of 13 mg. was obtained when 25 mg. of pituitary extract was given twice daily as compared with an average weight of 38 mg. when the same amount was

TABLE 1. THE EFFECT OF THE NUMBER OF DAILY INJECTIONS OF GONADOTROPIC PREPARATIONS ON THE OVARIES OF IMMATURE RATS

Kind of extract	Total dose[1]	No. rats	No. inject. per day	Av. wt. ovaries
				mg.
Sheep pituitary	25	6	2	13
	25	9	24	38
	50	6	2	17
	50	9	24	105
	100	9	2	35
	100	9	24	277
	200	6	2	62
	200	9	24	311
Sheep pituitary+heme	50+4 mg.	3	2	146
	50+4 mg.	9	24	309
FSH (*a*)	500	8	2	57
	500	3	24	249
	500	2[2]	24	85
FSH (*b*)	500	4	2	26
	500	6	24	61
	500	4[2]	24	44
Horse pituitary	10	6	2	16
	10	6	24	19
Beef pituitary	300	5	2	10
	300	5	24	199
Human PU	6 cc.	2	2	43
	6 cc.	6	12	46
	1.5 cc.	6	2	19
	1.5 cc.	6	24	18
PMS (untreated)	0.2 cc.	5	2	74
	0.2 cc.	6	24	64
PMS (purified)	0.2 mg.	5	2	43
	0.2 mg.	6	24	44

[1] In mg. equivalent of dry pituitary powder or cc. equivalent of urine.
[2] Hypophysectomized rats.

given 24 times a day. Two hundred mg. of the same extract when given twice daily resulted in an average ovarian weight of 62 mg. as compared with 311 mg. when it was injected 24 times a day. The ovaries produced by the hourly injection method usually contained both follicles and corpora lutea. The number of corpora lutea, however, was not great, and a few of the large ovaries contained follicles only. Although a quantitative study was not made, we believe that the number of follicles was greater in the ovaries which were produced by increasing the number of injections to 24 per day.

The follicle stimulating preparation [(*a*) table 1] prepared from sheep pituitary powder by a modification of the Fevold method (17) when given in a dose of 500 mg. equivalent twice daily produced ovaries which had an average weight of 57 mg. as compared with 249 mg. when administered 24 times per day. The same amount of this extract given 24 times a day to two hypophysectomized rats resulted in ovaries which had an average weight of 85 mg. The follicle stimulating preparation made by the trypsin method [(*b*) table 1] when given in a dose of 500 mg. equivalent twice daily produced ovaries which had an average weight of 26 mg. as compared with 61 mg. when given 24 times per day. When the same preparation was given to hypophysectomized rats the ovaries weighed 44 mg. These results show that hourly injections of follicle stimulating preparations as well as crude extracts of sheep pituitary powder, are more effective than injections made only twice daily.

The data in table 1 show that an increase in the number of injections of aqueous extract of horse pituitary powder did not produce ovaries heavier than those obtained by our standard method of administration of gonadotropic preparations, i.e., 9 injections in 4.5 days. Beef pituitary extracts, as well as extracts of sheep pituitaries, are greatly increased in their effectiveness by injecting on the hourly basis. The results show that an average ovarian weight of 199 mg. was obtained when an aqueous extract of beef pituitary powder was injected hourly as compared with an average ovarian weight of 10 mg. when injections were made twice daily for 4.5 days. The ovaries produced by increasing the number of injections contained many cystic follicles and relatively few corpora lutea. Analysis of the data in the accompanying table shows that horse pituitary, human pregnancy urine, and pregnant mare serum preparations did not become more effective when the number of injections was increased. McShan and Meyer (16) have reported that the effect of these gonadotropic preparations also is not enhanced when they are mixed with heme which greatly augments the action of sheep and beef pituitary extracts. Cole, Guilbert and Goss (19) have reported earlier that a single injection of pregnant mare serum into immature rats produced ovaries as large as those produced when injections were made twice daily for 4 days. At the present time there does not seem to be an adequate explanation for the fact that some gonadotropic preparations are more effective when mixed with augmenters or when the number of injections is increased. However, it does seem probable that a quantitative investigation of the elimination of such gonadotropic preparations by the kidney in relation to the method of increasing their effectiveness would be of value.

The relationship between the augmentation produced by heme and the increased effect obtained when sheep pituitary extract was given 24 times per day is recorded in table 1. The results show that 50 mg. of sheep pituitary extract plus 4 mg. of heme produced ovaries which had an average weight of 146 mg. as compared with 105 mg. when the same quantity of extract was given without heme 24 times daily. The most effective method that we have found for augmenting the effect of sheep pituitary extracts on the ovaries of immature rats is the hourly injection of a heme-gonadotropic mixture. Thus an average ovarian weight of 309 mg. was obtained when 50 mg. of pituitary extract plus 4 mg. of heme was given 24 times per day as compared with an

average ovarian weight of 146 mg. for the heme-extract solution given twice daily. These data show that the usual method of assaying sheep and beef pituitary gonadotropic preparations is very inefficient and does not give a true index of the potential activity.

The data obtained by increasing the number of injections or by using augmenters suggest that the increased ovarian weight is the result of making available to the ovary a constant, although small, amount of gonadotropic hormone. It seems probable that the hourly injections accomplish this because of the frequency of the administrations and the heme-gonadotropic mixture do so because the heme in some unknown manner causes the gonadotropic hormone to be released slowly from the injection sites. The most effective growth of the ovary, as influenced by gonadotropic hormones, is probably dependent upon the constancy as well as the amount of the gonadotropic hormone in the blood. A similar theory has been expressed by Bischoff (20). Large variations in concentration, such as exist when infrequent injections are made, are relatively inefficient in producing ovarian growth. It is suggested that the maintenance of the gonads in the normal animal is also dependent upon the continued secretion of minute quantities of gonadotropic hormone.

SUMMARY

Increasing the number of injections of sheep and beef pituitary gonadotropic extracts from 2 to 24 per day, resulted in a large increase in the effect of the extract on the ovaries of immature rats. This increased effectiveness was similar to that obtained when heme-pituitary gonadotropic solutions were administered twice daily. Maximum augmentation of sheep pituitary extracts was obtained by the hourly injection of heme-gonadotropic preparations. The increased effectiveness of gonadotropic preparations given in this manner is believed to be the result of the maintenance of a small but constant amount of the hormone in the blood.

The effectiveness of gonadotropic preparations made from pregnant mare serum, human pregnancy urine and horse pituitary powder was not increased by increasing the number of injections from two to twenty-four per day.

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ANTIHORMONE PRODUCTION TO CRUDE AND PURIFIED PREGNANT MARE SERUM PREPARATIONS

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ACKNOWLEDGE of the nature of the inhibitory substances produced in response to injections of various endocrine materials is necessary before the physiological significance of these so-called antihormones can be stated. Although many approaches to this problem have been employed, the question has not yet been definitely settled, and the reader is referred to the recent excellent reviews on the subject (1, 2). One method of attack, not sufficiently investigated, has been to determine the relative amounts of antihormone evoked by hormone preparations of different degrees of purity. Thus, for example, Rowlands and Young (3) have reported that administration to rabbits of a crude pyridine extract of ox anterior lobe resulted in the production of greater amounts of antithyrotropic substance than did a purified thyrotropic preparation. Such findings are interpreted as supporting the contention that the antihormones are antibody in nature. On the other hand, we (4) have recently shown that a greater inhibitory effect is obtained with antiserum to a purified pregnancy urine extract than with that produced to an untreated pregnant mare serum although the latter antiserum, even after specific absorption, yielded a greater precipitin reaction than the former. Although these results suggest that reactions other than those which are truly immunological in nature may also operate in the production of antigonadotropic substances, a complete solution of the problem is still forthcoming.

An obviously better approach to the question would be to examine the activity of antisera evoked by one type of hormone material purified to different extents. Through the kindness of Drs. Erwin Schwenk and Gerhard Fleischer, Schering Corporation, there was placed at our disposal a number of pregnant mare serum powders of different degrees of purity obtained at different stages of the extraction process. In this report, we present the results of a study designed to compare the relative amounts of antihormone produced in rabbits and rats by various pregnant mare serum preparations.

MATERIALS AND METHODS

In the first series of experiments 4 groups of adult male rabbits (3 in each group) were subcutaneously injected daily with 20 Cole-Saunders (c-s) units of the pregnant mare serum (PMS) preparations of different degrees of purity. The 4 preparations used assayed as follows: *Preparation A*, 1240; *Preparation B*, 440; *Preparation C*, 20; and *Preparation D*, an untreated PMS, 0.3–0.4 c-s units per mg. dried powder. The animals were bled from the heart after 2, 3½ and 5 months of treatment, and the sera obtained from rabbits injected with the same hormone preparation pooled and stored in a refrigerator for the duration of the experiment.

In the second series of experiments 20 adult Bartonella-free female rats were

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injected daily with 2 c-s units of a purified PMS preparation (Gonadin). Another group of 20 received daily injections of 2 c-s units of crude untreated PMS (veterinary Gonadin).[1] Half of the rats were killed after 50 and the other half after 110 days of injections. The sera of the animals in each of the 2 groups were pooled.

A total of 627 immature female rats (25–28 days) were employed to assay the various antisera. For the rabbit antisera, the test animals were injected daily for 5 days with varying quantities of the sera together with 3 c-s units of the specific PMS preparation against which the antiserum had been formed. Controls were injected daily for 5 days with hormone alone. The rat antisera were assayed by injecting the animals daily for 5 days with 0.3 cc. of the antiserum along with 1.5 c-s units of either the purified or crude sample. Controls received the same amount of hormone alone or the hormone along with 0.3 cc. normal rat serum. In all cases, the hormone was injected on one side, and the serum on the other side of the body to avoid mixing. All animals were killed on the 6th day, the ovaries carefully dissected, trimmed, dried on blotting paper and weighed on a torsion balance to the nearest milligram.

RESULTS

A. *Rabbit antisera.* It is seen from table 1 that the inhibitory capacities of the antisera *A*, *B* and *D*, obtained after 3½ months of injection were, on the whole,

TABLE 1. MEAN OVARIAN WEIGHTS ± STANDARD ERROR OF IMMATURE RATS INJECTED DAILY FOR 5 DAYS WITH VARYING QUANTITIES OF RABBIT ANTISERA, PRODUCED TO 4 PMS PREPARATIONS OF DIFFERENT PURITY, PLUS 3 C-S UNITS OF THE SPECIFIC PMS SAMPLE AGAINST WHICH THE ANTISERUM HAD BEEN FORMED. THE NUMBERS OF ANIMALS FROM WHICH THE MEANS WERE COMPUTED ARE GIVEN IN PARENTHESES.

	Rabbit Antisera							
Total Amt. Antiserum (cc.)	A		B		C		D	
	3½ mo.	5 mo.	3½ mo.	5 mo.	3½ mo.	5 mo.	3½ mo.	5 mo.
1.0		12.2 ± 1.1 (10)		13.3 ± 1.0 (10)	18.8 ± 0.6 (8)	21.1 ± 0.8 (11)	37.6 ± 2.2 (8)	78.7 ± 5.2 (11)
0.5	12.6 ± 1.0 (7)	15.6 ± 1.7 (11)	13.0 ± 0.5 (7)	20.3 ± 1.2 (10)	31.9 ± 1.9 (10)	33.8 ± 2.1 (11)	72.0 ± 4.2 (9)	85.2 ± 4.3 (10)
0.25	22.8 ± 1.5 (8)	28.3 ± 2.0 (11)	23.0 ± 0.7 (8)	29.0 ± 2.1 (12)	37.1 ± 2.2 (11)	36.6 ± 2.3 (11)	82.8 ± 6.3 (8)	94.2 ± 3.9 (12)
0.125	27.5 ± 1.8 (8)	34.1 ± 1.8 (11)	35.5 ± 1.6 (8)	42.1 ± 1.5 (10)	62.6 ± 4.3 (11)	46.3 ± 1.9 (11)	78.5 ± 5.2 (7)	102.4 ± 5.2 (10)
0.0625	40.0 ± 2.1 (7)	55.2 ± 2.4 (9)	53.3 ± 2.4 (8)	54.6 ± 2.2 (7)	90.2 ± 3.7 (11)	68.2 ± 2.2 (8)	83.5 ± 6.1 (8)	95.8 ± 5.1 (8)
0.03125	55.0 ± 1.6 (7)		68.0 ± 2.1 (7)					
0 (controls)	85.8 ± 3.8 (29)		87.9 ± 4.6 (17)		82.7 ± 3.2 (12)		97.8 ± 5.2 (21)	

slightly greater than those obtained after 5 months of treatment. On the other hand, the higher dilutions of the 5-month antiserum C possessed a slightly higher potency than the 3½-month C samples. The results for the 2-month antisera are not included in the table but were practically the same as those obtained after 3½ months of injection. It appears thus that a maximal amount of antihormone to PMS is formed in the rabbit as early as the 2nd month of injection and that treatment beyond this time does not materially affect the antigonadotropic content of the serum.

The data also indicate that for both the 3½ and 5-month periods of injection, the

[1] The PMS, in the form of clinical and veterinary Gonadin, was supplied by the Cutter Laboratories, Berkeley, California, through the courtesy of Mr. Donald Wonder.

inhibitory capacity of antiserum *A* was approximately equal to that of antiserum *B*. Both *A* and *B* were more inhibitory than *C* which, in turn, was more potent than *D*. Thus, for example, complete inhibition of the effect of 15 c-s units of hormone was accomplished by 0.5–1.0 cc. of antiserum *A* whereas this quantity of antiserum *D* pro-

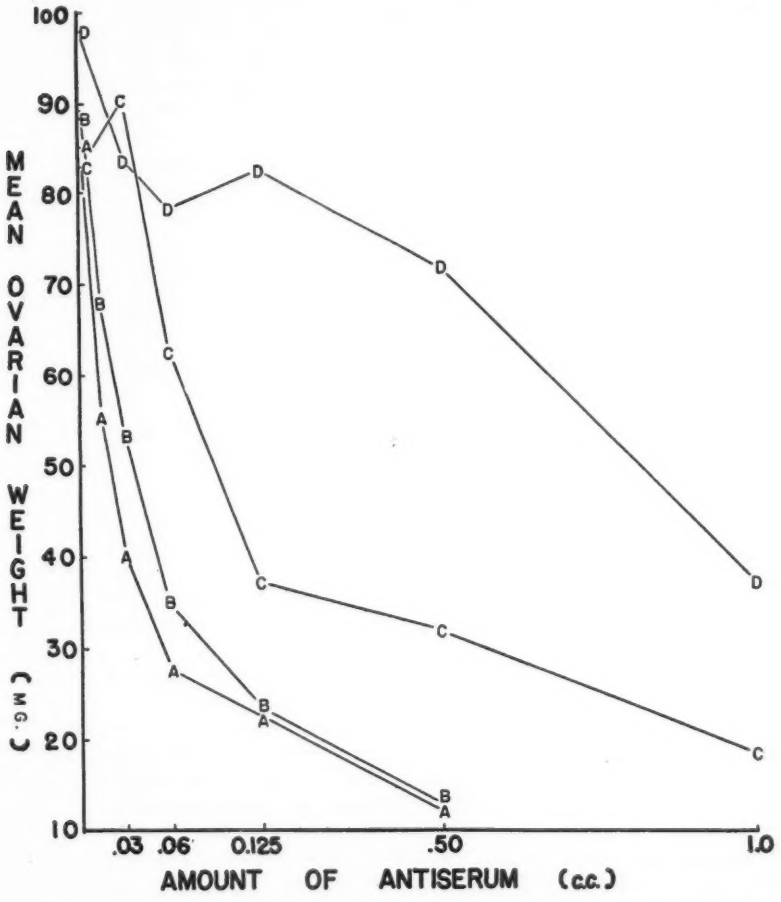

Fig. 1. Lines indicating the inhibitory capacities of 4 rabbit antisera formed to PMS *preparations A, B, C,* and *D* and obtained after 3½ months of daily injection. Each curve passes through the mean ovarian weights of immature rats injected daily for 5 days with varying quantities of these antisera plus 3 c-s units of PMS.

duced little or no effect on the same amount of hormone. Moreover, as little as 0.03 cc. of antiserum *A* or *B* exerted an inhibitory action whereas this was not obtained with the same quantity of antiserum *C*. It would thus seem that the greater the purity of the mare serum hormone preparation the more antihormone material evoked. This is true, however, up to a certain limit of purification. As can be seen, further purification of samples assaying of 400 c-s units per mg. (PMS *preparation B*) does

not appreciably change the amount of antigonadotropic substance produced. For convenience in making comparisons, the results for the 3½-month antisera are plotted in figure 1.

B. Rat antisera. The results with the rat antisera were substantially similar to those obtained with the rabbit antisera. Thus, as can be seen from table 2, the purified PMS caused a greater antihormone response in rats than the crude PMS. This was true for both the 50 and 110-day periods of injection. The 110-day antiserum *F*, evoked by purified PMS, was somewhat more potent than the 50-day sample. Antiserum *G*, produced by crude PMS, however, possessed approximately the same weak antihormone power after 50 and 110 days of injection. Data, not indicated in the

TABLE 2. MEAN OVARIAN WEIGHTS ± STANDARD ERROR OF IMMATURE RATS INJECTED DAILY FOR 5 DAYS WITH 1.5 C-S UNITS OF EITHER PURIFIED OR CRUDE GONADIN PLUS 0.3 CC. OF THE 50 OR 110-DAY RAT ANTI-PMS SERA. IN PARENTHESES ARE INDICATED THE NUMBERS OF ANIMALS FROM WHICH THE MEANS WERE COMPUTED.

	Rat Antisera			
	F (Produced to purified Gonadin)		*G* (Produced to crude Gonadin)	
Tested against	50-day	110-day	50-day	110-day
Purified Gonadin	22.1±0.8 (15)	14.4±1.3 (12)	33.1±2.2 (8)	31.2±2.8 (7)
Crude Gonadin	17.7±1.3 (8)	12.3±1.6 (8)	29.5±1.0 (13)	28.6±3.1 (19)

Controls	Purified Gonadin	Crude Gonadin
Hormone alone	39.7±2.0 (18)	37.9±1.8 (19)
Hormone±0.3 cc. normal rat serum	40.4±2.2 (10)	43.9±2.4 (11)

tables, have indicated that rat antisera are decidedly less inhibitory than sera obtained from rabbits injected with proportionate amounts of the same PMS material for the same period of time.

DISCUSSION

Perhaps the most interesting finding in this study is that the more purified PMS preparations evoke, in both rabbits and rats, a greater antihormone response than cruder samples. A number of explanations may be advanced for this phenomenon. One possibility is that untreated crude PMS possesses, in addition to the active hormone and the antihormone evocating factor (which may be the hormone itself), some substance which inhibits antihormone formation. This material could exist entirely independently or closely associated with the hormone, and might be present normally in blood or appear during pregnancy. Normal serum proteins are known to depress reticulo-endothelial activity and antibody production if administered in large enough quantities over long periods of time. The reticulo-endothelial system has also been shown to be intimately concerned with antihormones (5), and such factors may very well lower antihormone production. If this view is correct then the more purified PMS preparations, possessing less inhibitory substance, thus cause a greater antihormone response. Accordingly, the fact that rabbit antisera *A* and *B* possess about the same degree of antihormone capacity would imply that the factors which inhibit antihormone formation, if they do exist, are almost completely removed in the extraction process at a point where 1 mg. dried solid equals approximately 400 c-s units. Experiments designed to test the extent to which normal horse serum, serum albumen,

serum globulin and various pregnancy metabolites affect the antihormone response to purified PMS preparations are now in progress.

Apart from the existence of antihormone inhibiting factors, it may also be possible that the active hormone in the crude PMS is linked to some material and that this moiety, as a whole, is not as good an antihormone-evocator as another substance, derived from the first complex, which becomes more concentrated with repeated purification. In view of the high degree of purity of several of the PMS preparations employed in this study, it would seem that the factor which induces antihormone formation is either the hormone itself or some substance, non-hormonal in nature, but very closely related, in chemical properties, to it. The possibility that it is actually the hormone cannot, of course, be proven until the pure hormone is available, and its ability to induce antihormone formation tested.

It is difficult to reconcile our results with other experiments like those of Rowlands and Young (3) who have shown that crude pituitary extracts evoke more antithyrotropic substance than purified thyrotropic preparations. It should be borne in mind, however, that the principles inducing anti-PMS and antithyrotropic substances possess different properties and may be affected by different factors. This fact, for example, is supported by the finding that whereas longer periods of injection cause the appearance of greater amounts of antithyrotropic substance, anti-PMS factor production attains a maximum relatively quickly and remains at approximately the same level despite continued treatment. In addition, the 2 hormones are derived from different sources, and it is conceivable that materials produced specifically during pregnancy along with the PMS hormone could alter the course of the antihormone reaction to it.

It would also appear from this study that antihormone and orthodox immune body production do not necessarily proceed together. Antisera containing little or no antihormone but capable of inducing precipitin formation, even after specific absorption with normal horse serum, have been produced to crude PMS in the present and in previously reported experiments (4). Along the same lines, Rosen and Marine (6) have found that rabbits injected with iodothyroglobulin do not become refractory to the metabolic effect of this substance but nevertheless exhibit a definite immune response in the form of precipitins. Schulhof (7) has likewise demonstrated the failure of serum obtained from animals injected with thyroglobulin to antagonize the physiological effect of this substance.

One might conclude from this study that the antihormone-evocating factor in PMS is the actual hormone and, in addition, that the inhibitory substance produced to it is not immunological in nature since antihormone production increases with the removal of crude antigenic material from the injected hormone extract. Because of the limited quantity of hormone available, it was not possible to conduct extensive in vitro immunological experiments in the present work. The possibility, therefore, that the antihormone-evocating factor is itself a specific weak antigen or attached to some weakly antigenic carrier cannot, at the present, be completely excluded.

SUMMARY

Four PMS preparations of different degrees of purity were injected daily into rabbits for 2, 3½ and 5-month periods. The inhibitory capacities of these 3 sets of antihormone sera, produced to each hormone preparation, were approximately the same. In each case, however, for the 3 periods of treatment, the antisera formed to the more purified hormone preparations were more inhibitory. Similar results were obtained with antisera produced in rats injected for 50 and 110 days with crude and purified PMS preparations. Since the quantity of antihormone is increased by re-

moval of concomitant, non-hormonal material from the injected extract, it would seem that the antihormone-evocating factor in PMS is the actual hormone or some chemically related substance.

I wish to thank Drs. Irving Levenstein and Harry A. Charipper for aid and advice given during the course of the investigation.

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EFFECT OF HYPOPHYSECTOMY ON DIURNAL RHYTHM OF SPONTANEOUS ACTIVITY IN THE RAT

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THE FIRST RECOGNITION, from a modern scientific standpoint, of a diurnal rhythm in the albino rat was by Szymanski (1), who demonstrated and described a 24-hour cycle in the "voluntary" or spontaneous activity of the rat. Slonaker (2) previously noted that the major portion of the rat's activity, in normal illumination, falls in the period of darkness, i.e. at night. Szymanski (1) and later Slonaker (3) observed that despite minor fluctuations such as the "hunger cycle" of Richter (4), the rat is active for the 10 hours between, roughly, 8 P.M. and 6 A.M., and quiet for the major portion of the other 14 hours. Szymanski found that the total activity increased in rats maintained in constant darkness, and furthermore than the diurnal rhythm in activity persisted under such conditions. Thus the diurnal rhythm in activity of the rat may be classed as "persistent" under the definition proposed by Welsh (5). These conclusions have been corroborated and somewhat amplified (6).

With the exception of these papers, attention has, in the past, been directed mainly toward other cyclical aspects of spontaneous activity. The 2-hour cycle which parallels the hunger contraction of the empty stomach has been studied (4), as well as the 4- or 5-day cycle in activity paralleling the estrous cycle (7–9). In addition, Richter (10) has performed many experiments concerning the effects of various experimental procedures on the day-to-day level of spontaneous activity.

It has become well recognized that the level of spontaneous activity from day to day is dependent upon various endocrine influences. The gonads, thyroid, pituitary and adrenals have all been mentioned as possible controlling agents (3, 10–15). Intimately concerned with the influence of the various endocrine glands is the effect of light on diurnal rhythms in activity. Hemmingsen and Krarup (8), and Browman (9) have demonstrated several peculiar phenomena associating light, activity and the estrous cycle: (*a*) estrus always appears at the peak of the diurnal activity cycle; (*b*) reversal of illumination brings about a reversal of the diurnal activity cycle and a shift in the length of the next estrous cycle following the reversal, so that the actual time of estrus again coincides with the peak of activity; (*c*) constant light brings about constant estrus, with an accompanying decreased level of activity (16); (*d*) all these effects may be nullified by section of the optic nerve. This evidence seems to indicate some definite relation between the diurnal rhythm in spontaneous activity and the estrous cycle.

Jores (17) and Levinson (18) have demonstrated the presence of a 24-hour cycle in the melanophore hormone in the blood of the rat. Jores suggests that, as is the case in lower vertebrates, the melanophore hormone in mammals is the connection (Überträger) between light and the endocrine system.

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The experiments reported here were designed, therefore, to determine the specific role of the pituitary in the control of the diurnal rhythm in spontaneous activity in the rat.

APPARATUS AND PROCEDURE

The apparatus used in these experiments was a running-wheel equipped with an automatic recording system modified from those described by Hemmingsen and Krarup (8) and by Richter and Wang (19). A similar apparatus has been described by Park and Woods (20).

The experiments were conducted in sound-proof darkrooms, at a constant temperature of 73°F. In those experiments requiring illumination during some part of the day or night artificial light, controlled by a Tork clock, was supplied.

The animals were normally fed Purina rat food and water. Glucose was added to the powdered food supplied to the hypophysectomized rats. In many cases these operated animals also received 10% sucrose and 1% NaCl in their drinking water during the first 10 postoperative days. After this period they were again placed on the normal diet.

Two strains of rats were used in these investigations: the Sprague-Dawley albino strain, and an inbred half-wild strain bred by Dr. K. S. Lashley, to whom the authors are indebted for a supply of these animals. Before being hypophysectomized, all animals were placed in the running wheels for at least one week in constant darkness. In this manner, the diurnal activity following the removal of the pituitary could be directly compared with the normal rhythm in the same animal.

RESULTS

Behavior of normal animals. During the course of these experiments several interesting phenomena were noticed in the behavior of the intact animals. The albinos exhibited roughly from 1/4 to 1/10 as much total activity as did the half-wild stock. Moreover, the individual variation from animal to animal within either strain was considerable. In the albino rat it varied from zero to two or three thousand revolutions per 24 hours; in the half-wild rats, from 200 to 18,000 or more revolutions per 24 hours. In addition, some animals began to run immediately after being placed in the running-

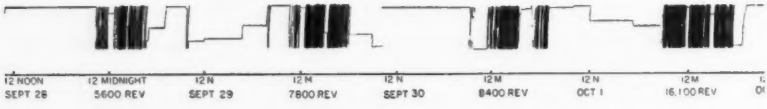

Fig. 1. TYPICAL NORMAL RECORD OF SPONTANEOUS ACTIVITY. (In this and the following figures, one set of records has been presented as typical for the group of animals under consideration.)

wheel; others did not run even after two weeks or more in the wheel. In general, a training period of from a week to 10 days was found necessary before a reasonable amount of activity was displayed on the wheel. All the animals which ran during the preliminary trials exhibited a diurnal rhythm in spontaneous activity similar to that described by Szymanski (1). A portion of a typical normal record is shown in figure 1. The diurnal rhythm, however, was more marked in the half-wild animals than in the albino rats.

It may be mentioned that some animals, especially of the albino strain which was used, showed little or no activity on the running-wheel; even after a conditioning period of 2 weeks, the amount of activity in some cases remained below 10 revolutions

per day. Of a total of 25 albino females, 14, or 56%, never ran enough to justify their use in this study.

All active females exhibited an increase in activity every fourth or fifth night—an indication, according to Hemmingsen and Krarup (8), of the night on which the animal comes into estrus. We have not taken vaginal smears of these animals, due to the undesirable stimulation of such a procedure.

The diurnal rhythm in spontaneous activity in the rat will persist for at least 5

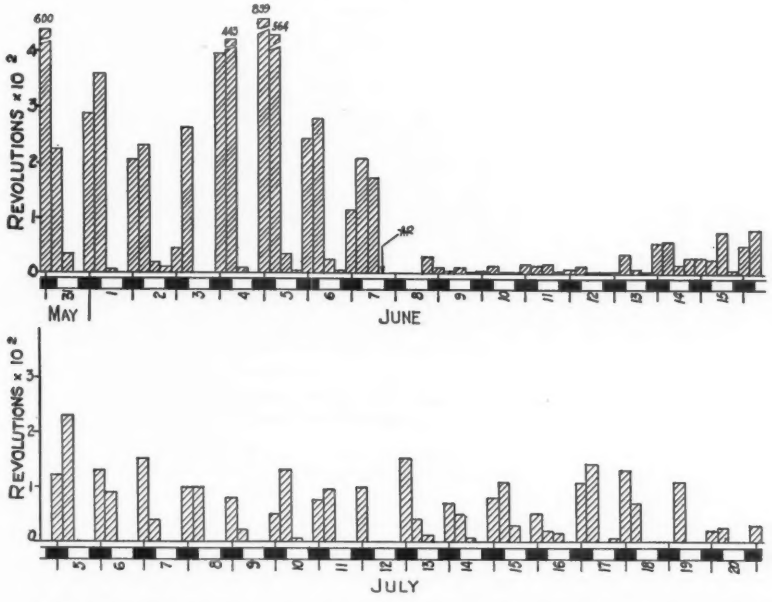

Fig. 2. Effects of hypophysectomy. The top graph shows the condition prior to and immediately after the operation. An interval of 20 days elapsed between this and the lower graph, which shows the same animal after final readjustment. The black areas below the abscissa indicate the period from 6 P.M. to 6 A.M. The experiment was carried on in total darkness.

months in darkness. Johnson (21) has demonstrated a persistent diurnal rhythm in the wild forest deer-mouse, *Peromyscus*, after 18 months in total darkness.

The effects of hypophysectomy. Thirty-seven animals were employed in these experiments; their disposition is described in the following protocol.

There were 37 animals which ran for some period of time on the running-wheels. Of these one was a male, 36 were females (including 11 half-wild and 25 albino animals). The one male, a half-wild, was run for 45 days, but was not hypophysectomized. Of the 11 half-wild females, one never ran, and on autopsy proved to have some abnormality of the endocrine system (exhibited in small ovaries, adrenals, etc.); one died of an intestinal obstruction; 7 were operated upon and died during the first two days; 2 were autopsied after 30 postoperative days, during which records were taken. Of the 25 albino females, 11 were hypophysectomized; 6 of these died within the first 2 postoperative days; 1 died on the tenth postoperative day, 1 on the 28th day, and one on the 50th day; the remaining two lived 72 days, then were autopsied.

The effects of hypophysectomy may be divided, for convenience, into three stages:

an initial stage, covering the first 2 days, a middle stage (second day to the middle of the second week), and a final stage (beginning by the 12th postoperative day). During the initial phase there is no significant amount of activity displayed on the running-wheel. The animals exhibit the usual postoperative inactivity and weakness. After this initial period there follows a middle stage during which the amount of activity per 24 hours gradually increases, and the resumption of the diurnal rhythm begins to appear. As the animal enters the final stage of readjustment, there is a

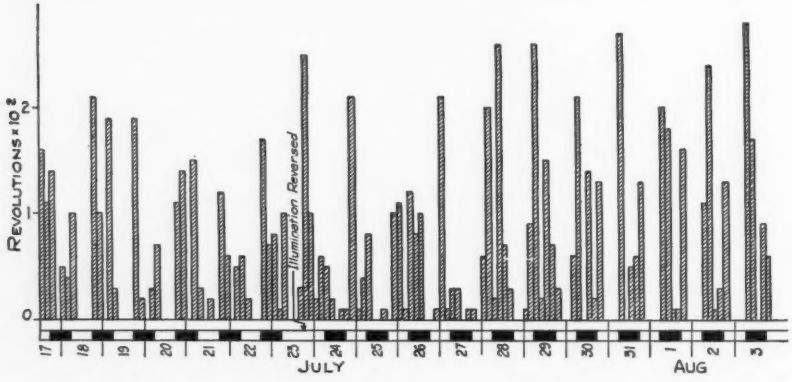

Fig. 3. REVERSAL OF THE DIURNAL RHYTHM IN SPONTANEOUS ACTIVITY IN HYPOPHYSECTOMIZED ANIMALS BY THE REVERSAL OF THE ILLUMINATION. The black areas below the abscissa indicate periods of darkness.

definite resumption of the diurnal rhythm in spontaneous activity. The total activity gradually increases, and within 30 to 40 days after the operation, the activity has reached a level one-third to one-half that of the preoperative level. A typical example of these changes is seen in figure 2.

Reversal of the diurnal rhythm in hypophysectomized animals. Since the diurnal rhythm became reestablished in hypophysectomized rats, it seemed desirable to attempt to reverse the rhythm by reversal of the illumination. Two animals, which had been hypophysectomized and had been maintained subsequently in total darkness for 41 days were subjected to a period of illumination from 6 A.M. to 6 P.M., simulating normal day and night, for 6 days. The hours of light and darkness were then reversed. For a period of 3 days following the time of reversal there was no change in the distribution of activity during the 24 hours. Following this period, however, there was a gradual shift and after 8 days, there was a complete reversal in the distribution of the activity (fig. 3).

DISCUSSION AND CONCLUSIONS

In addition to the experiments here reported, there have been at least three other descriptions of the relation of the hypophysis to diurnal rhythms. The earliest of these was that of Young (22) who found that the diurnal rhythm in color change in the lamprey was under the sole control of the melanophore hormone of the intermediate lobe of the pituitary. Removal of the pituitary was found to abolish the diurnal cycle in color change completely. In 1938 Jores (23) proposed, in continuation of his views of 1937 quoted earlier in this paper, that the pituitary "is the intermediary between light and the diurnal rhythm." Finally, Kalmus (24) has recently described a series of experiments on the larval axolotl. He found a persistent diurnal rhythm in

spontaneous activity in intact larvae, which lost its character of persistence in total darkness after hypophysectomy. There was no diurnal rhythm of any sort discernible in the metamorphosed adult. Final interpretation of these results must await further details and more extensive publication.

The authors have been unable to find any report of experiments describing the role of the pituitary in the maintenance of diurnal rhythms in mammals. Our experiments show that, in the rat at least, the presence of the pituitary is not essential to the maintenance of the diurnal rhythm in spontaneous activity. Hypophysectomy, however, does cause the cycle to become obscured for a period of about two weeks, but after this period, the diurnal cycle is reestablished. Furthermore the diurnal rhythm in spontaneous activity is reestablished at the time when the gonads, adrenals, thyroid and other endocrines, dependent upon the pituitary for their maintenance, are becoming definitely involuted, and becomes more and more evident when these endocrine glands have become atretic. Nevertheless, the pituitary is definitely involved in the maintenance of the day-to-day level of spontaneous activity since the level of activity after the removal of the pituitary is but one-third to one-half that prior to hypophysectomy. In this respect our results corroborate in general those of Richter (10).

In view of these results it is concluded that the presence of the pituitary gland is not essential to the maintenance of the diurnal rhythm in spontaneous activity in the rat. Whether or not it may be involved in the maintenance of this rhythm in the intact animal is a question which these data do not allow us to solve. The fact that two weeks must elapse before the diurnal activity is completely reestablished in hypophysectomized rats might suggest that some other mechanism, normally dormant or functional only to a minor degree, has taken over a function usually that of the pituitary. On the other hand, this lag of two weeks is also explainable on the basis of surgical shock and general endocrine imbalance.

SUMMARY

Twenty rats (11 albino and 9 half-wild) were hypophysectomized after preliminary adjustment to the activity recorder in continuous darkness and the securing of records of normal spontaneous running. During the first two postoperative days, all animals exhibited little activity and no diurnal rhythm. During the next ten days the amount of activity increased and the rhythm reappeared. After the second week, there was further increase in total activity, to $\frac{1}{3}$ to $\frac{1}{2}$ that of the preoperative level, and complete restoration of the diurnal rhythm.

Two hypophysectomized animals, 41 days after the operation, were exposed to periods of illumination corresponding to solar day and night for six days. The periods of illumination were then reversed, resulting in a corresponding reversal of the diurnal activity rhythm of the animals, as would have occurred in intact animals.

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OCCURRENCE OF OVARIAN "TUMORS" IN SPONTANEOUS VIRILISM OF THE HEN

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EMBRYOLOGICALLY, the hen's ovary is bisexual. The first proliferation of the ovarian germinal epithelium forms the primary sexual cords which later develop into the *rete ovarii*. The latter is not only a homolog of the testis, but can actually develop into one in certain circumstances. In the adult hen, the components of the *rete ovarii* can persist in a modified form in the atrophic right ovary and possibly in the left ovary. In the later developmental stages of the embryo the primary sexual cords are transformed into the following ovarian components (1): distended tubules with a large lumen and low epithelium, clusters or cords of fat-laden cells of Nonidez (also called luteal cells), isolated medullary cord cells, cords of primordial germ cells, cords of primordial germ cells bound together by a basement membrane, and mixed cords consisting of germ cells and fat-laden cells. Although these structures are characteristically those of the female, it has been reported that the developing sex cords may differentiate into seminiferous tubules in cases of sex reversal in hens (2, 3). The second proliferation results in the formation of the ovarian cortex and the follicular apparatus.

Left ovariectomy in the hen results in the development of a testis-like gonad in approximately 98% of the cases on the site of the right ovary, which is ordinarily atrophic in the normal hen (4). If the operation is performed early enough, spermatogenesis generally also develops. The administration of anterior hypophyseal gonadotropic hormones or the hormones of pregnant mare's serum likewise induces masculinization in the hen (5, 6, 7, 8).

Spontaneous sex reversal in birds is generally characterized by a masculinization of the female, although the development of female characteristics by a genetically determined male has been reported in a few sporadic cases. This discrepancy between the two sexes may be due to the fact that there is no rudiment in the testis of the ovarian cortex which is the specific source of the female sex hormone. There are undoubtedly organs other than the ovary which secrete estrogens and presumably in the male such a source might be the adrenal cortex. On the other hand, the homolog of the testis, usually in a functionless state, does remain in the medulla of the ovary. One may conclude from the embryology of the ovary and testis, therefore, that the female is more liable than the male to a certain degree of intersexuality in the development of its gonadal organs.

A number of investigators have studied the social behavior, secondary sex characteristics and ovarian pathology in spontaneous sex reversal of hens (2, 10–16). A survey of this literature shows that the gonads and accessory sex structures of masculinized hens are the seat of certain characteristic histological alterations. The ovarian tissue and oviducts are usually atrophic. In some instances the left ovary is destroyed and largely replaced by hemorrhagic cysts or widespread tuberculosis. In association with this pathology there have been found all

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possible transitions from the histological structure of a normal ovary to that of a typical testicle. Among the most characteristic of these histological findings have been irregular cords of polyhedral cells and undifferentiated tubules. Differentiated tubules have also been described in every phase of sperm formation. Indeed, in one of Crew's cases of sex reversal, a hen became the father of two chicks (16).

Previous investigations have dealt with the embryological and genetic aspects of this problem. In our own studies we have used the available embryological data to aid in the interpretation of our histological observations in order to arrive at an understanding of the pathological physiology which is involved. Because of the suggestive parallelism between masculinizing ovarian tumors of women and the ovarian tissue overgrowths found in masculinized hens, a comparative study of the pathology of these tissues was made.

MATERIALS AND METHODS

Eight yearling hens and pullets were obtained from a nearby farm in Massachusetts. Three of them were entirely normal in every way (a White Leghorn yearling hen, a White Leghorn pullet, and a Rhode Island Red pullet). The other 5 animals had certain of the physical characteristics of virilism (2 White Leghorn yearling hens with spurs, 1 White Leghorn pullet with spurs, and 2 Rhode Island Red pullets with male comb and wattles).

The hens were autopsied carefully and their pituitaries, adrenals, ovaries and thyroids were weighed and then preserved for sectioning (4–5μ) and staining. The hypophysis, thyroids, ovaries and ovarian tumors were fixed in Helly's fluid or 10% formalin, the adrenals in Orth's fluid. Sections of the hypophysis were stained by the modified Azan technic which differentiates the carmine cells originally found in the pituitaries of rabbits and cats (17, 18). The adrenal tissue was stained by the Broster-Vines' technic and Masson's polychrome stain. Both methods revealed about the same number of fuchsinophilic cells, but the latter gave better differentiation of the nuclear and cytoplasmic structures of the adrenal tissue.

OBSERVATIONS

Normal Controls

These 3 birds were fertile hens of Rhode Island Red and White Leghorn stock weighing between 1.7 and 2.5 kg. They appeared to be normal in every respect. At autopsy the endocrine organs were examined carefully to establish a standard for comparison with the virilistic hens.

Ovaries. The left ovary of the laying hens was large and contained many follicles of varying size; while in the non-laying hens, the left ovary was small and contained relatively few and poorly developed follicles. Corpora lutea and atretic follicles were also visible macroscopically. Nothing unusual could be seen histologically. Ovarian tissue was not found on the right side. The oviducts were large in the laying hens, much smaller in the non-laying hens.

Adrenals. Due to a distinctly lobular structure, it is difficult to differentiate clearly between the various zones of the hen's adrenal. The cortical tissue appears as irregular cords between which the medullary tissue interdigitates intimately throughout the whole gland. The cortical cells stain differentially either with the Broster-Vines or Masson technics. Most of these cells, which are long and cylindrical (round or polygonal in cross-section), take a gray-greenish color with the Masson trichrome method and contain a varying amount of vacuoles (? lipoids). The nuclei of these pale cells are fairly large and contain nucleoli. The fuchsinophil cells which are stained differentially with the Broster-Vines technic or Masson's polychrome stain, are less numerous

than these pale gray-green cells, and their relative number varies somewhat. The gray-green cells frequently contain some fuchsinophilic granules, although this reaction is usually sharply limited to the fuchsinophil cells. In many glands the periphery contains dark fuchsinophilic cells without vacuoles. In such instances the granules are so numerous that the other structures are almost indistinguishable. A more complete description of these cells has been made by Uotila (19).

Thyroid. The epithelium was relatively inactive with occasional secretory vacuoles and lymphocytic infiltration.

Pituitary. In addition to the usual cell types, the carmine cells (17, 18) were easily identified in all of the normal animals (fig. 1).

Spontaneous Virilism of the Hen

Hen A. This hen had some of the physical characteristics of the rooster. She had no spurs, but the feathering of the neck, and her carriage were distinctly masculine (fig. 9). Her history is unknown except for the fact that she had ceased to lay eggs, although at one time she was normal in this respect. Her weight was 2.7 kg.

Autopsy findings. The left gonad was smaller than in laying lens and the ipsilateral oviduct was atrophic. The ovary contained two large follicles about 1.5 cm. in diameter and many smaller ones. The right ovary could not be found. Near the hilum of the left ovary, and attached to it by a broad pedicle, there was a yellowish-white tumor about 1 cm. in diameter.

In the periphery of the tumor, which was surrounded by a connective tissue capsule, there were occasional groups of pigmented epithelial cells similar to those seen in the normal cortex of the ovary. The greatest part of the tumor was made up of irregular epithelial cords composed of fairly large cells (fig. 2) which had no membrana propria. Cells similar to these were also scattered without obvious pattern throughout the connective tissue of the tumor. In addition there were groups of similar epithelial cells arranged in a tubular form with a membrana propria. No spermatogenesis could be observed. The epithelial cells were polyhedral and undifferentiated; and the size of the nuclei varied considerably. Many cells contained one or more vacuoles of different size. The tumor was rich in hyalinized connective tissue. Surrounding the epithelial cords and atypical tubules were degenerative changes with localized and scattered lymphocytic infiltration.

Hen B was a White Leghorn with a relatively large comb and wattles and small spurs; she no longer laid eggs. Weight was 2.1 kg.

Ovarian tissue could not be recognized macroscopically and the left oviduct was atrophic. At the usual site of the ovary in the midline there was a brownish-red soft tumor mass, 2.5 × 0.7 cm. in diameter, which was partly attached to the left adrenal gland. Adherent to the anterolateral aspect of this elongated tumor was a round yellow-brown mass, 0.5 cm. in diameter. Caudal to the latter and contiguous with the first tumor was another yellow-brown mass, 1 cm. in diameter.

A few small degenerated follicles were found in the periphery of the elongated mass on microscopic examination. This tumor was composed of a peripheral epithelial portion (the cortex) and a highly vascular central core of connective tissue (the medulla). The cells of the germinal epithelium, which invaginates frequently, were arranged in a pseudostratified layer and occurred in thicknesses of one or more. The cortex of the tumor consisted of a lobulated mass of undifferentiated somewhat elongated epithelial cells which had a poorly defined cytoplasm, and oval to round fairly vesicular nuclei with one or more nucleoli. Occasional mitotic figures were encountered. Among these cells were small rounded clusters of slightly larger cuboidal to polyhedral cells with a moderate amount of fairly granular cytoplasm and oval nu-

clei with well scattered chromatin (fig. 3). Some of these clusters were arranged in a definite tubular structure with a typical membrana propria. Structures similar to the distended medullary tubules and composed of cubical and cylindrical cells were often seen in the cortex.

The second tumor was composed of a main mass, around the periphery of which were some degenerated and atrophic ovarian follicles. The main mass consisted of irregularly distended tubules, similar to medullary tubules, lined with cuboidal epithelium; and irregularly outlined cell cords with many undifferentiated epithelial cells made up the rest of the tumor. Proliferation of the cuboidal epithelium of the medullary tubules resulted in many instances in formations which are somewhat similar to the intracanalicular form of adenoma mammae (fig. 4). These intracanalicular structures were not medullary tubules which had broken loose from the membrana propria in fixation, because they had no membrana propria and the intracanalicular growth was obviously continuous with the cellular lining of the tubules in many instances. The periphery of the tumor was more solid than its central portion and consisted of irregular epithelial cell cords or epithelial cells without special arrangement.

Atrophic and cystic follicles were found in the periphery of the third tumor. This mass was composed of solid undifferentiated epithelium supported by fine reticular connective tissue. Numerous small and medium-sized distended medullary tubules occurred in the central portion of the tumor; there were fewer of these structures in the periphery (fig. 5). It was evident that the epithelium of the distended medullary tubules, or that which lay between them, had proliferated extensively. Because of the increased pressure the medullary tubules had been flattened. There was some mitotic activity in the numerous centers of proliferation of unorganized growth with pressure atrophy of the surrounding cells. Large degenerative epithelial areas were found outside the centers of proliferation. Many cells contained large fat vacuoles.

Hen C. A white, rooster-like hen with large comb and wattles and big pointed spurs (fig. 9); weighed 2.3 kg.

The right ovary could not be identified; the left one was atrophic. Microscopically the cortex of the left ovary was abnormally thin; and the follicles were unusually small and atrophic. The left oviduct was likewise atrophied.

In the center of the left ovary was a dark, red-brown fluctuating hemorrhagic cyst, which contained about 25 cc. of fluid and was the size of a hen's egg. A few atrophic ovarian follicles were found in the surface of the cyst. Three tumor masses were dissected from the inside of the wall of the cyst.

The yellowish tissue of tumor 1 seems to have been ovarian tissue originally. The characteristic pigmented cells were noted in its periphery. Scattered, particularly in the periphery, were structures somewhat similar to distended medullary tubules which were the seat of extensive degenerative changes. There was also an irregular growth of epithelial cords and scattered groups of one to three cells broken up everywhere by pink-staining homogenous areas characteristic of hyaline degeneration (fig. 6). The structure of this tumor is somewhat similar to that found in *Hen A* (fig. 3).

The second, and larger yellow-green tumor, measuring 1.5 × 1.5 cm., also contained the characteristic pigmented cells in its periphery. This finding in addition to the fact that it was located inside an ovarian cyst suggests the ovarian origin of this tumor. In a small area we found compact epithelial tissue in which polyhedral cells occurred in small clusters supported by fine reticular connective tissue (fig. 7). However, the major portion of this tumor was composed of cells whose cytoplasm was replaced for the most part by one large or a number of small vacuoles which left only a thin rim of cytoplasm around the periphery of the cell. The nuclei of these cells were shrunken and degenerative changes were common. In most of them there were very

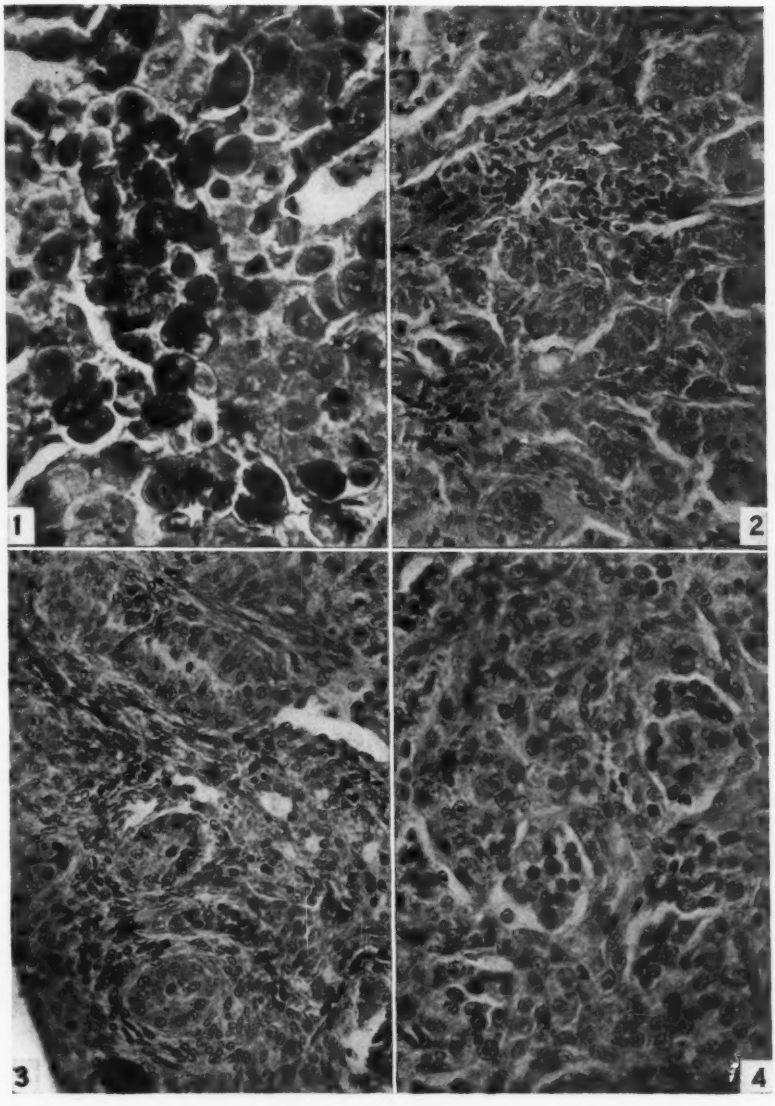

Fig. 1. Section from anterior pituitary of virilistic *Hen A*. The dark cells with the heavily stained granules are carmine cells. Some large basophils and smaller eosinophils are also visible. Formalin fixation, Azan stain. ×1025. Fig. 2. Ovarian tumor from virilistic *Hen A*. Cell cords and atypical tubules can be seen. The connective tissue is partially hyalinized. Helly's fixative. Hematoxylin and eosin. ×325. Fig. 3. Ovarian tumor from virilistic *Hen B*. Seminiferous-like, sterile tubules are seen under the peritoneal epithelium. One tubule is darkly stained and degenerating. Helly's fixative. Hematoxylin and eosin. ×325. Fig. 4. Ovarian tumor from virilistic *Hen B* of the so-called intracanalicular type. Notice the connection of intracanalicular epithelium with the epithelium of the walls of the tubules which are probably distended medullary tubules. Helly's fixative. Hematoxylin and eosin. × 485.

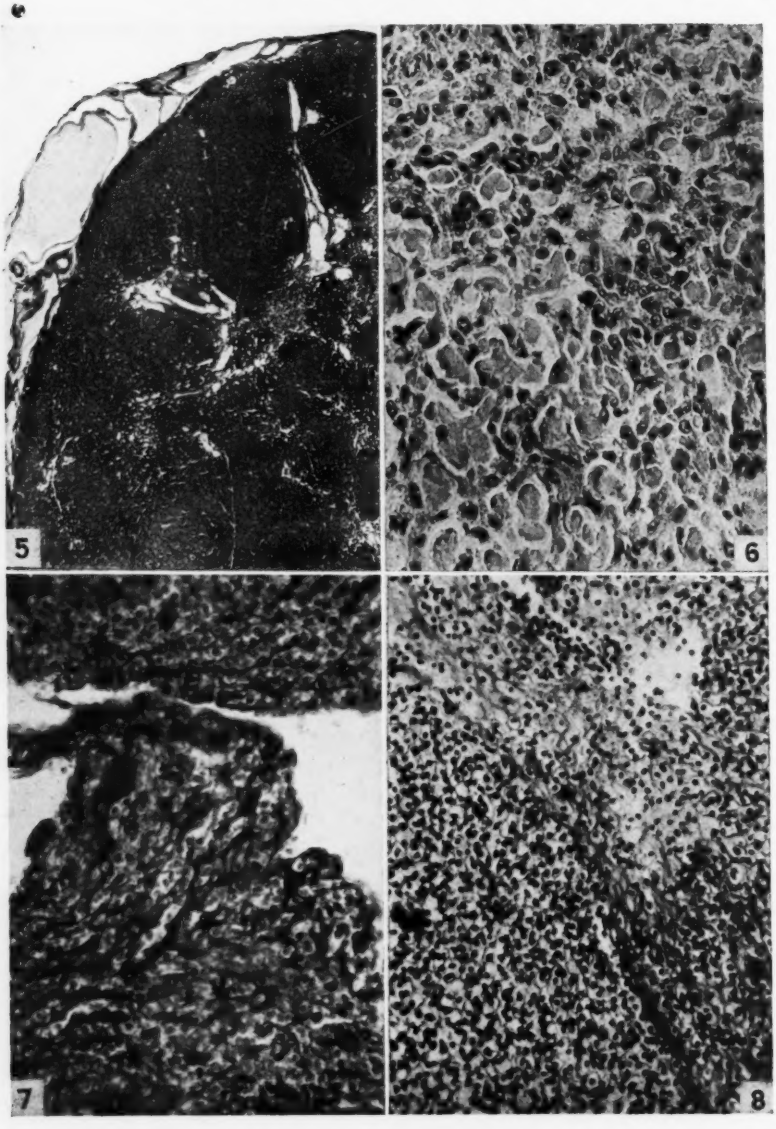

Fig. 5. OVARIAN TUMOR FROM VIRILISTIC *Hen B*. Atrophic and cystic follicles are visible at the periphery. In the central portions of the tumor there are formations similar to the distended medullary tubules. Their epithelium is cuboidal and proliferating. The light areas are centers of proliferation. Helly's fixative. Hematoxylin and eosin. ×28. Fig. 6. SECTION FROM MASS OF TISSUE FOUND INSIDE THE HEMORRHAGIC OVARIAN CYST IN VIRILISTIC *Hen C*. The epithelial cells occur singly and in cords. There is some similarity to figure 2 in the arrangement of cell cords and atypical tubules. Connective tissue largely hyalinized. Formalin fixation. Hematoxylin and van Gieson. ×425. Fig. 7. SECTION FROM ANOTHER MASS OF TISSUE INSIDE THE SAME CYST MENTIONED UNDER FIGURE 6. There are small cell cords consisting of 2 to 4 cells and separated from each other by fine connective tissue fibrils. The tissue in the upper part of the figure bears some resemblance to a macrocellular type of ovarian tumor. (cf. figure 8.) Formalin fixation, Ponceau-fuchsin

small fat droplets between the vacuoles; while many of them contained very large, partly anisotropic fat droplets. No glycogen was detected in any of the cells. Although reticular in some places, the connective tissue was for the most part a structureless, homogenous, hyalinized mass. In many areas it separated the cells widely from each other. There were but few blood vessels visible.

The third tumor consisted of a tissue similar to that of the second mass, but the cells were arranged in larger cell cords. The histological similarity of these three tumors and their topographical relations suggests that they were parts of the same tissue which had undergone different degrees of degenerative change.

Hen D. A Rhode Island Red non-laying hen had relatively large comb and wattles; no spurs, but masculine feathering of tail and neck. Weight was 2.1 kg.

The left ovary was atrophic, but contained numerous follicles, 2 or 3 mm. in diameter. At the lower end of the ovary, attached to it by a thick pedicle, there was a firm mass weighing 9 gm. (2.5 × 3 cm.). It was composed of an old hemorrhagic clot and a grey-brown tumor. The right ovary could not be found and the left oviduct was atrophic.

Large areas of the tumor contained the typical features of tuberculosis: giant cells, cheese-like necroses, fibrosis, lymphocytic and plasma-cell infiltrations and hemorrhages. The oval epithelial cells of approximately one-half of this tumor (fig. 8) were similar to those found in *Hen C*. In those portions of the tumor where these cells were well preserved, the cytoplasm contained many small vacuoles. In many instances the cytoplasm was almost totally replaced by a large vacuole. The nuclei were small and shrunken and one could not determine their structure. As in *Hen C* most of the cells contained small fat droplets which were partially anisotropic. Glycogen was not found in the cells.

The connective tissue occurred either in the form of a fine reticulum or consisted of large hyalinized septa. There were few blood vessels.

Hen E. A Leghorn, with large comb and spurs, did not lay eggs at the time of observation, but had done so in the past. Weight was 1.5 kg.

The left ovary was small; the right one absent. The left oviduct was atrophic. The cortical layer of the left ovary was very thin and contained several follicles measuring 3 to 4 mm. in diameter. The granulosa cells in some of these follicles were degenerating. At the caudal end of the ovary a firm, small, yellowish-white tumor measured 3 mm. in diameter. The tumor was composed of a rounded mass of apparently fairly dense fibrous tissue. The cells, which appeared to swirl, were small, closely packed, elongated, and accompanied by much collagen. No mitotic figures are seen. The number of cells was greater in the vicinity of the blood vessels than usually seen in a fibroma. Nothing of a pathological nature other than this tumor would be found.

Observations on the Pathology of the Adrenals, Thryoid and Pituitary in Masculinized Hens

Adrenals. The average weight of the adrenal glands removed from virilistic hens was uniformly and considerably less than that of the adrenals in the control group of animals (table 1). The fuchsinophil cells were present in both groups of hens although we have the impression that they were fewer in number and that this staining reaction was more feeble in the adrenals of the masculinized hens than in the normal ones.

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and anilin blue. ×520. Fig. 8. SECTION FROM THE TUBERCULOUS HEMORRHAGIC OVARIAN CYST IN VIRILISTIC *Hen D.* The cells in this tissue resemble the macrocellular type more closely than those in figure 7. The vacuolated cells contain pyknotic nuclei which are pushed to one side. The connective tissue is partially of fine fibrillar and partially of hyalinized material. Numerous degenerative changes are present. Formalin fixation. Masson's trichrome III. ×370.

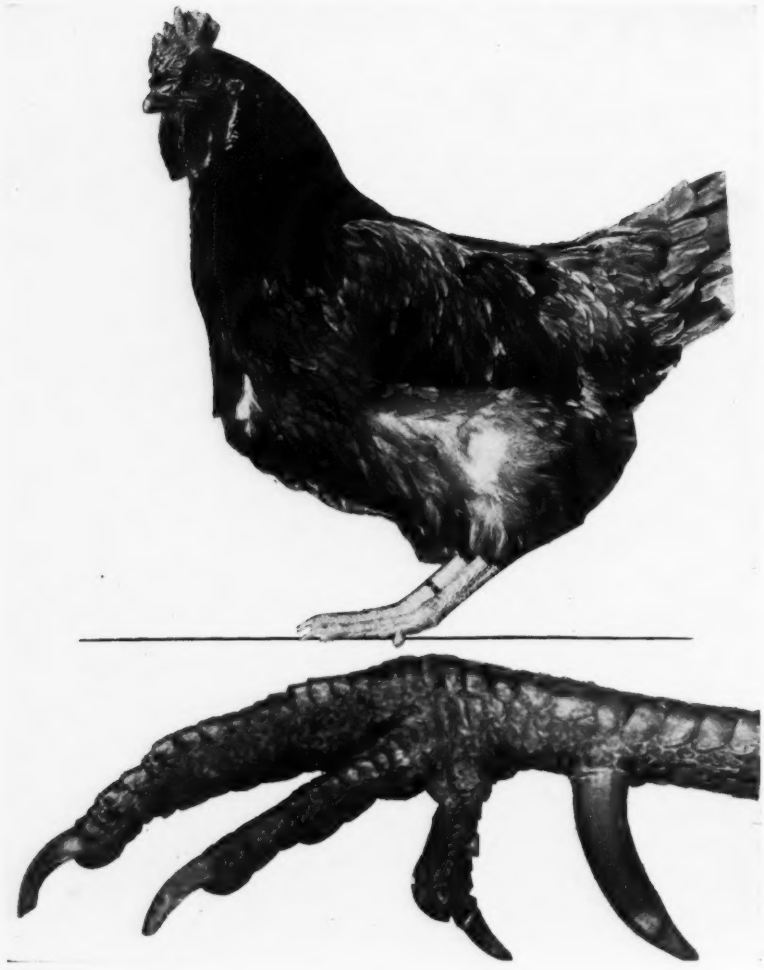

Fig. 9. *Hen A.* Note comb and wattle development, posture and feathering. The spurred foot is that of *Hen C.*

The marked difference in weight between the two groups of adrenals is too consistent to be fortuitous; its significance, however, is for the present a matter of pure speculation. According to Herrick and Torstveit (30) there is a definite interrelation between testes and adrenal function in the fowl. They observed that the testes and combs of adrenalectomized chickens (supported by cortin and salt) suffered severe atrophy fairly rapidly after operation. They questioned whether reduced testicular activity was the sole explanation for the decrease in size of the comb. In surveying the possible relation of the atrophied adrenals to gonadal function in our masculinized hens one must take into consideration at least two circumstances: the loss of ovarian function due to atrophy or destruction of the ovarian cortex, and the unnatural activ-

ity of androgenic tissue in a genetically determined female fowl. It is probable that either one or both of these disturbances are responsible directly or indirectly for the partial atrophy of the adrenals which has been observed.

Thyroid. The weight of the thyroids was more consistent in the group of virilistic hens than in normal ones; but the average weight was essentially the same in both groups of animals (table 1). Microscopic examination of the glands removed from the virilistic animals showed a relatively inactive gland with low cuboidal epithelium and concentrated colloid.

TABLE 1. WEIGHTS OF THE ADRENAL, THYROID AND PITUITARY GLANDS IN NORMAL AND VIRILISTIC FOWL

	Adrenals		Thyroid	Pituitary
	Right	Left		
Normal control hens	*mg.*	*mg.*	*mg.*	*mg.*
	110	92	206	10.0
	112	80	84	10.0
	112	95	102	13.5
	123	165	165	12.0
	160	140	206	8.0
Average	123	114	153	10.7
Virilistic hens				
A	83	53	187	11.9
B	84	—	184	17.0
C	75	93	180	9.5
D	80	70	129	11.0
E	80	—	126	12.0
Average	80	72	161	12.2

Pituitary. The pituitaries of the masculinized hens were found to be somewhat larger than those of the normal control hens (table 1). A detailed study of the cytological appearance of the anterior pituitary has been deferred for the present. It was noted, however, that the so-called carmine cells described by Friedgood and Dawson (17, 18) were to be found in all of the glands.

DISCUSSION

Ovarian pathology in spontaneous sex reversal of hens. All 5 hens had in common the complete or marked atrophy of the left ovary. In one instance, hemorrhage (*Hen C*) and in another, tuberculosis (*Hen D*) was responsible for the destruction of the left ovary; in the 3 remaining hens, atrophy of the left ovary could not be attributed to any destructive pathologic process.

Histologic studies of the tissues at the site of the left ovary in all 5 hens disclosed compact masses of tissue which, for want of a better descriptive term, have been called tumors. The structure of the latter was found to be of 3 types: (*a*) more or less differentiated tubules and cords of cells (*Hens A* and *B*), (*b*) compact epithelial tissue composed of large vacuolated polyhedral cells which contain fat droplets (*Hens C* and *D*); (*c*) a dense fibroblastic tissue composed of small, elongated cells which tended to be arranged in whorls (*Hen E*).

Closer examination of the tissues of the first type discloses the following structures: (*a*) distinct sterile seminiferous-like tubules (*Hen B*, tumor 1); (*b*) irregular cords of cells with but few tubule-like formations, (*Hen A*); (*c*) distended tubules with intracanalicular extension of the epithelial wall (*Hen B*, tumor 2); (*d*) compact epithelial tissue with distended tubules (*Hen B*, tumor 3). From a histological viewpoint the

'tumors' in *Hens A* and *B* have much in common with the arrhenoblastomata. According to Meyer (20) the arrhenoblastoma is a tumor which has some of the morphological characteristics of testicular tissue and which secretes a biologically active androgen. Meyer has somewhat arbitrarily divided these tumors into 3 groups morphologically. He points out, however, that even in the same tumor there may be found quite different types of tissue structure. In the cases reported in the literature it has been found that the morphological appearance of different tumors, and even of different parts of the same tumor, has varied from undifferentiated sarcoma, through that of poorly developed epithelium and irregular epithelial cords or masses, to that of rudimentary tubules or gland-like structures. This tumor apparently follows a general morphological pattern which is similar to that characteristic of various phases of the early development of the sex gland. This leads one to the reasonable conclusion, as Norris (21) puts it, that "pathogenetically, the arrhenoblastoma should be regarded as a malignant ovarian tumor whose morphologic appearance more or less corresponds to certain, some, or all of the varying structural conditions found in the indifferent stage of the sex glands' development." Meyer's attempt at morphological classification was apparently dictated by a desire to reconcile structure and function. The evidence suggests, however, that there is no justifiable reason for the subdivision of this group of tumors on the basis of their histologic structure alone. It is clear, however, that Meyer has made a most important contribution in calling attention to the derivation of the arrhenoblastomata from embryological rests of undifferentiated ovarian tissue and in emphasizing that these tumors secrete a biologically active androgen. This interpretation of his data is especially interesting not only because the ovarian masculinizing tumors of our virilistic hens have many histological features in common with the ovarian medulla of chick embryos of young pullets, but also in view of the fact that similar masculinizing 'tumors' can be produced experimentally by stimulation of the ovarian medulla with the anterior hypophyseal gonadotropic hormones.

The compact epithelial 'tumors' composed of large vacuolated polyhedral cells which contain fat droplets (*Hens C* and *D*) resemble closely the so-called macrocellular masculinizing tumors (grosszellige Vermännlichungstumoren) in man. The differential diagnosis of the arrhenoblastomata from these macrocellular tumors has offered considerable difficulty (22, 23, 24). The latter are solid tumors whose large, vacuolated cells are embedded in a delicate connective tissue network, or, as in Sellheim's case, in broad connective tissue cords. The cytoplasm contains varying amounts of lipoids, some anisotropic, and the cell nuclei are small, shrunken and polymorphic. Meyer has withheld judgment in regard to their relation to the arrhenoblastomata chiefly because they lack tubules. Pathologists competent to judge have placed them in various categories from a pathologic viewpoint. Some believe that they are related to the hypernephromata; others consider them lutein cell tumors; still others classify them with the arrhenoblastomata (20, 22, 24–27).

The tumor in *Hen E* differed from all the others in that it did not contain epithelial tissue. It is difficult to conceive of the cellular elements of this tumor in a secretory capacity, but it should be recalled that the structural appearance of certain arrhenoblastomata has been likened to that of an undifferentiated sarcoma (21). It may be significant that the number of cells in the vicinity of the blood vessels of this hen tumor was greater than one expects to find in an ordinary fibroma.

It is apparent that atrophy of ovarian tissue or its surgical ablation or destruction by disease (hemorrhage or tuberculosis) are precipitating factors which are common to hens undergoing sex reversal. It is clear also that ablation of a major portion of ovarian tissue by one or more of these means stimulates a new growth of tissue on the site of

the original ovary or in the medulla of the atrophic right ovary. This new growth is composed essentially of seminiferous-like tubules and cords of cells which in all probability are responsible for the secretion of the masculinizing hormone. One may conclude that there is a consistent and close parallelism between the ablation of ovarian tissue and the growth of a testis-like tissue in the spontaneously occurring as well as in the experimentally produced cases of sex reversal in the hen. This parallelism suggests that a similar mechanism is responsible in both circumstances for the phenomenon of ovarian masculinization. The available experimental evidence indicates that the anterior pituitary is an intermediary factor in the pathogenesis of such sex reversal.

It has been well established that the administration of anterior pituitary gonadotropic hormones to pullets can induce sex reversal (5–8). Uotila (9) has confirmed and extended such observations. That there is a hyperactive state of the pituitary in our virilistic hens is quite probable for the following reasons. Castration is known to result in the increased production, storage and secretion of the gonadotropic hormones of the anterior pituitary. What amounts to complete castration has been found to be the only factor common to all of the virilistic animals; and it has been noted, furthermore, that the pituitary is generally somewhat hypertrophied in this series of cases (table 1). Histological studies of the ovaries of pituitary injected pullets or of the regenerated right ovary after left ovariectomy in adult hens indicate that the increased production of male sex hormone, which is responsible for the sex reversal, stems from an hypertrophied ovarian medulla in each instance.

The transference of conclusions from one species to another without serious reservations is hazardous. Nevertheless, one may speculate on the pathological physiology of the arrhenoblastomata in view of their similarity in pathology to the masculinizing hen tumors. Certainly this seems to be a reasonable viewpoint from which to undertake a further study of this problem. With this in mind, it is pertinent to enquire into the condition of the uninvolved ovary in cases of arrhenoblastoma, especially because an atrophy or the pathological destruction of ovarian tissue is the initial incident in the cycle of events ending in virilism of the hen. Reports in the literature which touch upon this point agree that the uninvolved ovary is smaller than it should be for the age of the patient and that it is commonly atrophic (31). Phelan (28) found the uterus and the left tube and ovary in a 20-year-old girl so atrophic that he was tempted to remove them for their museum value. Taylor, Wolfermann and Krock (29) state that in an 18-year-old girl the uninvolved right ovary was about half the usual size and without the slightest evidence of Graaffian follicles or corpea lutea. The uterus in this case was about 1/3 normal size. Norris (21) autopsied a 34-year-old woman who died of an arrhenoblastoma and found that the non-neogenic ovary appeared atrophic and more like that of a much older woman. He decided, on the basis of histological studies, however, that suppression of ovarian function was far from complete. Nevertheless, he was impressed by the atrophic condition of the uterus, mammary glands and ovary. The significance of this ovarian atrophy from an etiologic viewpoint is still a matter for discussion and speculation. Our own interpretation of the situation is based on the observations in the human being that complete removal of the arrhenoblastoma prior to its metastasis results in a return to normal size and function of the other ovary, uterus and mammary glands (21). This fact leads us to conclude that when an arrhenoblastoma in one ovary is functionally active, the opposite ovary is either directly or indirectly suppressed physiologically; whereas, with the removal of this depressant influence, the uninvolved (and apparently undamaged) ovary is permitted to respond again to the normal stimuli which are necessary for its proper function. If this reasoning is correct, the situation differs from that which we have described for the mecha-

nism of sex reversal in the fowl in which a primary atrophy or the pathological destruction of ovarian tissue is the stimulus which indirectly initiates the growth of the hen 'tumor.'

It is hoped that our observations will lead to a renewal of the interest in this field which was first evinced by the geneticists and embryologists who studied sex reversal in the fowl without considering the possible clinical significance of their investigations.

SUMMARY

Studies of the ovaries of 5 hens in whom spontaneous sex reversal had occurred revealed complete pathologic destruction or marked atrophy of the left ovary. The adrenals of these animals weighed considerably less than those removed from comparable normal individuals. Histologic studies of the tissues at the site of the left ovary disclosed compact masses of tissue which, for want of a better descriptive term, have been called 'tumors.' The histological structure of these tumors was essentially identical with that of testicular tissue in various stages of differentiation. The pathology of certain of these hen tumors distinctly resembled that of the arrhenoblastomata of women.

Available evidence indicates that the growth of the hen tumors was induced by the pathologic destruction or atrophy of the left ovary (equivalent to complete castration in the hen). It is thought that the latter was responsible for hypertrophy of the anterior pituitary gland and that the resultant increase in secretion of the gonadotropic hormones stimulated the vestigial male-sex-hormone-producing cells of the atrophied medulla of the ovary to become functionally active.

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VAGINAL ESTRUS IN IRRADIATED MICE

RELATIONSHIP OF VAGINAL ESTRUS TO OVARIAN CHANGES AND PRODUCTION OF BIOLOGICALLY ACTIVE OVARIAN TUMORS AFTER ROENTGEN IRRADIATION

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IN A PREVIOUS COMMUNICATION, the experimental production of biologically active ovarian tumors in mice by roentgen irradiation was reported (1). Granulosa and theca cell neoplasms with varying degrees of luteinization were found to occur in a number of mice during periods of 10 to 15 months following irradiation. Histogenetically, these tumors appeared to have been derived from undifferentiated ovarian parenchyma. Their estrogenic hormonal activity was expressed in proliferative changes within the endometrial and vaginal mucosa. Since these tumors were comparable in structure and function to the granulosa cell neoplasm, its excessively luteinized variety, the Lecène tumor, and the theca cell tumor of the human ovary, an analogous derivation from ovarian parenchyma was postulated.

In the course of this study, animals were killed at frequent intervals during the many months prior to tumor development, permitting a correlation between the histopathology of the ovary, the uterine endometrium and the vaginal mucosa. It was noted that despite complete destruction of the follicular system by x-ray, full estrous smears were obtained in many instances. Particularly, therefore, for the light it might cast upon the hormonal function of the ovary altered by irradiation, we have attempted in this investigation to study vaginal estrus in mice from the time of irradiation to senescence or tumor formation.

It has been previously shown that the x-ray obliteration of the follicular system in the mouse ovary does not inhibit the subsequent development of accessory organs in the immature animal, nor cause their atrophy in the adult (2). Parkes, Fielding and Brambell (3) have noted also that the estrous cycle continues after x-ray sterilization. These authors, on the basis of a few months follow-up, concluded: (*a*) that in mice irradiated at weaning time puberty occurred at the usual time (6 to 7 weeks of age) and continued with normal periodicity, (*b*) that mice sterilized at an earlier age tended to show a more abnormal cycle, and (*c*) that in x-rayed adult mice, the cycle persisted quite unchanged, with only slightly increased variability in length. Schught (4) examined the vaginal smears of adult mice 6 months after x-ray castration, and found irregular estrus in 11 out of 19 animals. Guinea pigs (5) and rats (6) examined during a period of several months after irradiation showed similar results.

It was obvious from these investigations that the Graafian follicle was not the only source of the estrus producing hormone, since after x-ray destruction of the follicle apparatus, sufficient estrogens were elaborated to produce extraovarian changes of estrus. These phenomena ceased, however, after bilateral oöphorectomy,

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suggesting that the remaining parenchyma of the ovary was responsible for the estrogenic stimulation.

For a proper evaluation of vaginal estrus after roentgen irradiation a brief resume is presented of the ovarian changes in mice following x-ray exposure. Arbitrarily these histologic changes may be divided into four stages, which are not distinctly demarcated, but merge gradually, one into the other.

(1) *Stage of follicle destruction.* In mice irradiated at puberty the primary oöcytes and the ova of the larger follicles are quickly destroyed. The granulosa cells degenerate progressively, but more slowly. The theca interna cells and interfollicular tissue begin to show evidence of luteinization during the 3rd and 4th week after irradiation. By the 6th to 8th week, few remnants of the follicular system remain, and the ovaries present a picture of almost complete luteinization.

(2) *Stage of luteinization.* The ovary, though normal in size, is replaced by groups of enlarged, polygonal or rounded cells with distinct, vesicular nuclei and abundant cytoplasm, containing doubly refractile fat. Ovaries examined during the 3rd, 4th, 5th and 6th month show few conspicuous changes from this picture beyond variations in the degree of luteinization and evidence of some degeneration in groups of luteinized cells.

(3) *Stage of proliferation of the surface epithelium.* After the 6th month an additional phenomenon is noted. This consists of a surface proliferation which invades the cortex of the ovary in the form of tubular or adenomatous epithelial strands. Though at first confined to small crescent or thin layer at the periphery, it becomes more conspicuous with age. During this stage the medullary portion of the ovary still contains luteal tissue, part of which presents large cells with distinct, vesicular nuclei and abundant, foamy cytoplasm, while other portions show varying degrees of plasmolysis.

(4) *Stage of tumor formation or senesence.* In the second year after irradiation biologically active tumors may replace the ovaries or the latter shrink in size and gradually become replaced by inactive, dark staining, epithelial cells having occasional glandular arrangement.

METHODS AND MATERIALS

All the mice employed in this experiment were female, white and derived from homogeneous stock. At the time of irradiation they were approximately 6 to 7 weeks of age (puberty) and had begun to show estrus. A single dose of 200 r., measured in air, was given over the animal's trunk, with the head adequately shielded from the rays by lead. The irradiation factors were: 180 kv., 8 ma., 50 cm. distance and 1.0 mm. Cu filtration.[1]

Twenty-three mice were subjected to the roentgen rays as indicated above, and segregated. Vaginal smears, using a small pipette and a drop of saline, were taken daily on each mouse for a period of 14½ months. The mechanical stimulation of vagina and cervix was carefully avoided by extreme gentleness during this procedure. Those animals that died spontaneously during this time were examined immediately to insure the minimum of post-mortem change. Complete autopsies were made. The ovarian, uterine and vaginal sections were correlated with the vaginal smears and the preceding courses of estrus.

The vaginal smears were stained with an alcoholic solution of basic fuchsin after preliminary drying. Only those smears showing non-nucleated, cornified squamous cells exclusively were considered to present a full estrous reaction. A mixture of non-nucleated squamous cells, nucleated epithelial cells and occasional leucocytes was interpreted as indicating a diminished or subestrous stimulation. Smears showing less stimulation were classified under diestrus.

As a control for the irradiated animals, 25 female mice of similar age and stock

[1] The irradiation was carried out in the Department of Radiotherapy of the Mount Sinai Hospital through the kindness of Dr. William Harris and with the cooperation of Dr. Louis Zaretski.

were followed. Vaginal smears were taken at regular periods to note any change in the duration of and interval between estrus in the non-irradiated mouse. One mouse was killed each month for comparison with the autopsy findings in the x-rayed group.

RESULTS

The results of daily vaginal smears taken on 23 mice over a period of 14½ months after irradiation is represented in figure 1.

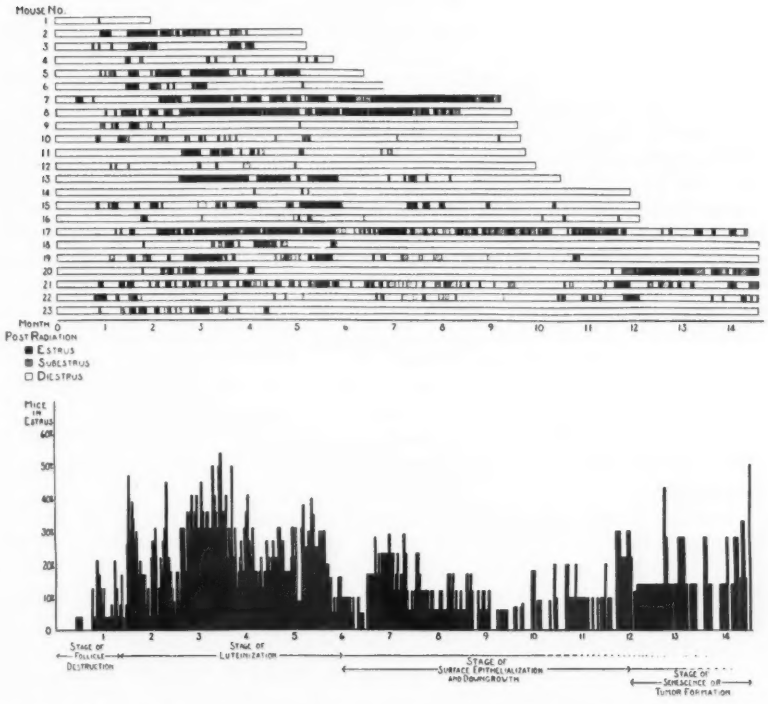

Fig. 1 (above). Occurrence of vaginal estrus in irradiated mice as indicated by daily vaginal smears over a period of 14½ months. Following a temporary abolition of estrus immediately after irradiation, the estrous cycle returns in a totally irregular fashion, with marked variations in the duration of continuous estrous periods (1 to 63 days) and the intervals between estrus (1 to 222 days).

Fig. 2 (below). Correlation of the daily percentage of vaginal estrus with the ovarian changes following the roentgen irradiation of mice. A reestablishment of estrus is noted with the onset of luteinization of the ovarian parenchyma, which gradually declines during the latter half of the first year. With the development of biologically active ovarian tumors, however, increased estrogenic secretion is again expressed in irregular, prolonged estrous periods.

No evidence of estrus was found in any of the mice but one, from the day following irradiation until the 24th day. This period corresponded with the stage of follicle destruction within the ovaries, and suggested that the rapid destruction of the follicle system removed the chief source of estrogenic stimulation of the vagina.

By the third to the fourth week after x-ray, approximately one-third of the cases had begun to show estrus, and at the 10th week, a positive vaginal smear was obtained in all but one of the mice. It is to be noted that the first histologic evidence of

luteinization of the ovarian parenchyma was observed between the third and fourth week after irradiation and that this change became fairly complete throughout the ovary by the eighth week. This time relationship between the onset of estrus and the beginning of luteinization would seem to indicate that the newly formed lutein cells, through the elaboration of an estrogenic substance, restored estrus in the irradiated mouse.

The course of estrus was found to be totally irregular, and entirely unlike its regular, cyclical occurrence at 4- to 7-day intervals in normal control mice. Estrus was observed in each mouse at irregular intervals and for varying lengths of time. The longest, continuous period of estrus was found to be 63 days (*mouse 7*). Many mice showed occasional subestrus or threshold smears, suggesting a diminished effect, insufficient to give a full estrous smear. As illustrated in figure 1, the mice differed in the extent and duration of estrous stimulation.

After the sixth month, during the stage of surface epithelial proliferation and down-growth in the ovary, luteinization within the medullary portion of the ovary could still be seen with, however, increased evidence of degeneration and decreased hormonal function. While irregular estrus was present in 100% of the mice in the first 6 months after irradiation, only 37% of the mice gave evidence of this phenomenon in the second 6 months, and then to a lesser degree. The ovaries of those mice with prolonged diestrus during the latter part of the first year after irradiation gave evidence of progressive replacement by proliferating surface epithelium. In those, however, who continued to show estrogenic activity, the functional capacity of the luteinized cells apparently persisted.

At the end of the 14½ months period of observation, 7 surviving mice were killed. *Mice 18, 19* and *23*, in whom vaginal smears had indicated persistent diestrus for many months preceding death, presented quiescent ovaries. The others, in whom estrous smears were obtained at this late date, showed the presence of granulosa cell neoplasms.

In attempting to reconstruct the sequence of events, it would appear that the initial suppression of estrus is dependent upon the x-ray destruction of the ovarian follicles, and possibly also upon a temporary inhibition of the incretory powers of the ovarian parenchyma. Then, under gonadotropic stimulation, in all probability, the remaining inter-follicular tissue undergoes conspicuous luteal changes, which secrete estrogenic hormone in sufficient amount to cause prolonged stimulation of the vagina, as manifested in totally irregular vaginal estrus. Later, the occurrence of a diminution in estrous phenomena in some mice may be related directly to degenerative changes within the luteinized ovary, and perhaps to decreased gonadotropic stimulation. After one year, a recurrence of prolonged, acyclical estrus is dependent upon the development of biologically active granulosa cell tumors, composed of lutein cells, cytologically similar to those found in the early luteinized ovary.

SUMMARY

The effects of roentgen irradiation upon the estrous cycle of female mice were studied in 23 animals by daily smears over a period of 14½ months. The changes noted within the vagina reflect those within ovaries and are described in correlation with them.

(1) *Stage of follicular destruction.* The destructive effects of x-ray upon the ovarian follicular system of female mice in the first 4 to 8 weeks after irradiation are accompanied by a temporary abolition of estrus during that time.

(2) *Stage of luteinization.* The lutein changes within the residual ovarian parenchyma which then develop result in the elaboration of sufficient estrogenic hormone

to reestablish vaginal estrus in 100% of the animals. The estrous cycle is found to be totally irregular, with marked variations in the duration of continuous estrous periods (1 to 63 days), and the intervals between estrus (1 to 222 days). Individual differences are apparently related to the degree and duration of functional activity within the luteinized ovarian parenchyma.

(3) *Stage of surface epithelial proliferation and downgrowths.* From 6 months to one year after irradiation, surface epithelial proliferation and downgrowth replace a good portion of the ovarian cortex, with diminution of luteinized tissue and increased evidence of degenerative change. During this time, the extent of vaginal stimulation is found to be decreased and the number of mice showing acyclical estrus is reduced from 100% to 37%.

(4) *Stage of senescence or tumor formation.* After one year following irradiation, a prolonged absence of estrus may be correlated with senescent changes within the ovary. Those mice, however, which develop biologically active, luteinized granulosa or theca cell tumors once more exhibit increased estrogenic secretion expressed in irregular, prolonged estrous periods.

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EFFECTS OF LARGE AMOUNTS OF ANDROGEN ON THE TESTES OF THE PREPUBERAL RAT[1]

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THERE HAVE BEEN numerous reports that androgens have an inhibitory effect on the testes of the intact rat (1–4). However, Cutuly and his coworkers have presented evidence that this effect is not obtained with all dosages (5, 6), i.e., very large doses of androgens, instead of inhibiting the testes, maintain them at a normal size. This evidence seems to have been neglected by other workers, perhaps because it happened to be incidental data included in studies on another subject. It correlated well, however, with the well-known fact that androgens in similarly large doses apparently had a direct effect on the testes of hypophysectomized rats and could maintain them at a normal size (7, 8). It seems logical to believe that the dosages used by other workers in the intact rat (which were lower than those used by Cutuly et al.) were sufficient to depress the hypophyseal gonadotropic function but insufficient to maintain the testes. Thus a diminution in testicular size resulted. While the dosage used by Cutuly et al. probably also depressed hypophyseal gonadotropic function, it was in itself sufficient to maintain the testes in the absence of hypophyseal stimulation. Selye and Friedman (9) also demonstrated that a very high dosage maintains testes at normal size, while relatively small ones depress them. More recently Shay and his coworkers (10) and Rubinstein and Kurland (11) have reached conclusions which contradict this concept and which also contradict each other. In view of the apparent controversy we have decided to present the data which we have accumulated on this subject. Part of our data seems particularly pertinent since it appears probable that Shay et al. have repeated an error which we had committed.

PROCEDURE

The male rats of 7 litters, 10 days of age, were selected for the experiment. Fifteen were treated with testosterone propionate[2] daily for 20 days, the remaining 15 were untreated and served as litter-mate controls. All animals were killed at 31 days of age, on the day following the last treatment. The testosterone propionate was injected subcutaneously in peanut oil. In an attempt to compensate for increasing body weight, the dosage was increased at 5-day intervals. For example, animals receiving a total of 25.0 mg. over the entire treatment period (table 1, *Sp 11* and *12*) were given 0.5 mg. daily for the first 5 days, 1.0 mg. daily for the second 5, 1.5 mg. the third 5 and 2.0 mg. the final 5 days. Those receiving a total of 140.0 mg. (*Sp 9*) were given daily dosages of 4.0, 6.0, 8.0 and 10.0 mg.

At autopsy the sexual organs were removed in toto and fixed in Bouin's fluid.

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[1] Supported in part by a grant from Ciba Pharmaceutical Products, Inc.

[2] Testosterone propionate in the form of Perandren, was supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

Forty-eight hours later the testes were dissected free from extraneous tissue and weighed.

After completion of this procedure, Zondek's article on the percutaneous absorption of sex hormones was published (12). Since we had caged treated and non-treated litter mates together, it seemed very probable that the control animals had been contaminated, particularly since strong concentrations of androgens had been used. Accordingly 12 animals of the same age (31 days) and weight range were killed and used for normal control data. The same procedure was employed in obtaining the weights of their testes and these weights were used as controls for the experimental results previously obtained.

RESULTS

In table 1 the mean testicular weights for each of the treated and litter-mate control groups are shown. In each case the mean testicular weight was greater for the

TABLE 1. EFFECTS OF ANDROGENS ON TESTICULAR WEIGHT

Litter	No. treated	No. controls	Total dose	Body weight	Testicular weight	Mg. testes / gm. body weight
				gm.	mg.	
Sp 10 & 11	4		25.0	55.5	419	7.55
		3	—	50.7	254	5.02
Sp. 12	4		50.0	55.4	393	7.09
		4	—	43.3	193	4.45
Sp. 7	2		60.0	71.5	626	8.77
		2	—	64.5	363	5.60
Sp. 8	2		100.0	71.5	4.58	6.42
		3	—	74.0	367	4.96
Sp. 9	3		140.0	59.7	304	5.09
		3	—	63.3	188	2.97

treated animals than for the "theoretically untreated" litter mates. When corrected for body weight this same relationship was still obtained. In table 2 this increase is shown to be statistically significant (critical ratios greater than 3).

These findings, however, do not justify the conclusion that the testosterone propionate stimulated the testes to greater than normal weight. This is obvious when the data on the "clean" controls (individually caged) is considered (table 2). The gross testicular weights and corrected testes weights of the "clean" controls were not significantly different from those of the treated animals. However, the testes of the litter mates of the treated animals were significantly smaller than those of the normal animals. The small amount of testosterone propionate absorbed percutaneously (from the leakage of the highly concentrated androgen solution used for treated animals in the same cage and possibly from their excreta) caused a definite diminution in the size of the testes of these contaminated litter mates.

DISCUSSION

The data obtained in these procedures agree with those of Cutuly and Selye and

TABLE 2. ANALYSIS OF DATA

	Body weight				Testicular weight				Testes wt./gm. body wt.			
	Mean	σ	Crit. ratios		Mean	σ	Crit. ratios		Mean	σ	Crit. ratios	
			Lit. cont.	Cl. cont.			Lit. cont.	Cl. cont.			Lit. cont.	Cl. cont.
Treated animals (15)	60.4	2.26	0.67	1.93	422	29.8	4.05	0.43	6.96	0.12	4.16	0.64
Litter-mate controls (15)	57.7	3.32		0.50	266	24.6		4.16	4.59	0.29		6.68
Clean controls (12)	55.8	1.85			406	22.9			7.26	0.28		

σ=Standard deviation=$\sqrt{\frac{\Sigma d^2}{N(N-1)}}$

Critical ratio=$\frac{\text{obtained difference}}{\sqrt{(\sigma_{m_1})^2+(\sigma_{m_2})^2}}$

Significant differences underlined (3.00 or more).

their coworkers. Large doses of androgens maintain the testes at a normal size. They do not, however, agree with those presented by Shay and his coworkers, and by Rubinstein and Kurland.

In table 3 data on the testicular weights of prepuberal animals published by various workers are listed together with data of our own. The ratio of testicular weight to body weight (mg. testis per gm. body weight) has been calculated from data presented by the authors. As would be expected, there is some variation in the animals of different colonies. However, the ratios obtained from the data on the control animals of Shay and his coworkers (1.94 and 2.52) are very much smaller than those of

TABLE 3

Authors	Controls		Treated		
	Age	Mg. test. gm. b. w.	Treatment	Age	Mg. test. gm. b. w.
Shay et al. (10)	"31" (9)[1]	1.94	5 mg. 6×week	"31" (12)	4.27
	25 (3)	2.52	1 mg. 3×week	25 (3)	2.99
Rubinstein & Kurland (11)	32 (10)	7.57	2.5 mg. T.P. daily intraperitoneally	32 (10)	9.14[2]
Moore & Price (2)	31 (2)	10.60			
	32 (4)	9.07			
Greene & Burrill	21 (14)	6.34			
	31[3] (12)	7.26	25–100 mg. in 20 days	31 (15)[3]	6.96
	31 (9)[4]	8.90	And. contam. controls	31 (15)[3]	4.59
	35 (12)	7.78	0.1 mg. daily	31 (9)[4]	2.71
	39 (7)	7.14			

[1] No. of animals in group given in parentheses.
[2] Wt. of testis plus epididymis.
[3] Weighed after fixation—all other groups fresh weights.
[4] Previously reported groups (16).

any age in any other colony (6.34 to 10.60). It is unlikely that inter-colony variability could account for such relatively minute testes in supposedly normal animals.

In explanation of this descrepancy it may be noted that Shay et al. stated "littermate controls were used throughout." Although they do not specifically state that these control litter mates were kept in the same cages with treated animals, it seems possible that such a procedure was employed. This may have led to the same error which we originally committed: i.e., the supposedly untreated controls were contaminated by percutaneously absorbed androgens in amounts which produced a diminution of testicular weight. The testicular stimulation in prepuberal animals with very high doses of androgens claimed by these authors was therefore possibly due to the fact that their "control" litter mates were not actually normal control animals.

Additional evidence that the supposed normal control testes of Shay and his coworkers are not normal is furnished by their photomicrographs (fig. 2, 4). These photomicrographs supposedly represent testes from a normal prepuberal animal (29 days old). However, there is evidence of testicular damage in these testes (degeneration of germinal epithelium, small, shrunken tubules with some almost empty (fig. 2).

Rubinstein and Kurland conclude, among other things, that the highest dose they used (2.5 mg. testosterone propionate daily) caused a significant depression in the testicular weight of their prepuberal rats (−17%). The mean body weight of this treated group was 55.6 gm. compared to 60.8 gm. for the controls. This difference in body weight (9.3%) they state is not significant. However, if the data on the testicular weights of these 2 groups are corrected for body weight, i.e., compared on the basis of mg. testes per gm. body weight, the obtained ratio for the treated group is only 5.09% smaller than the control ratio instead of the "significant" 17% calculated by the authors. It is impossible to calculate from the presented data the statistical significance of this 5.09% depression.

Even if the corrected value for the testicular depression were significant, it is doubtful that it would have much bearing on the subject. In all of the groups treated by these authors with "high" doses the testosterone propionate was administered intraperitoneally. Deanesly and Parkes demonstrated in 1937 (13) that intraperitoneal testosterone propionate has little androgenic effectiveness. For example, 2.0 mg. testosterone propionate administered subcutaneously to castrate adult rats over a 10-day period causes a combined prostate-seminal vesicle weight of 385 mg. The same dose administered intraperitoneally in the same solvent over the same period of time causes a combined prostate-seminal vesicle weight of only 51 mg., which is barely more than castrate level.

Since testosterone propionate loses much of its effectiveness when administered intraperitoneally, the results obtained by Rubinstein and Kurland with this method are equivalent to those obtained with relatively small doses given subcutaneously. This could account for any testicular depression which they may have obtained.

These authors do not quote Deanesly and Parkes, but contend that testosterone propionate does not lose effectiveness when given by the intraperitoneal route. The evidence they cite for this contention is that "the seminal vesicles and testes of our two groups were roughly in the same weight class after treatment." The 2 groups referred to are those which received 0.02 mg. testosterone propionate daily, one by the subcutaneous route and the other intraperitoneally. Since the "testes weights" of their intraperitoneal group were actually the weights of the testes and epididymides, while those of the subcutaneous group were testes weights without epididymides, this comparison is not very valuable.

As a matter of general principle, it seems that the weighing of combined testes and epididymides and considering the result as testicular weight would add nothing

but confusion to a study of this type. Below a certain level the androgens would depress testicular weight, but at the same time would stimulate the weight of the epididymis. These opposed effects could hardly add accuracy to the results which these authors use as the basis for their quantitative conclusion on the effects of different doses of androgens (some given subcutaneously and some intraperitoneally) on the testes.

In common with Moore and Price (3), Rubinstein and Kurland found that the higher doses of testosterone propionate cause progressively less testicular inhibition. These latter authors explain this finding in a complicated manner. "As the dosage is still further increased the gigantic proportions of the reacting secondary sex organs become so striking that much of the injected hormone is utilized merely for their maintenance. This diminishes blood androgen concentrations, thereby depressing to a lesser degree the anterior lobe of the pituitary. The testis is correspondingly spared." Since it is known that androgens have a direct stimulating effect on the testes in the total absence of the hypophysis, and that relatively high doses are necessary for this effect, their explanation (for which there is little or no evidence) seems cumbersome and unnecessary. A more direct and simpler explanation is that the higher doses of testosterone propionate have an increased direct stimulatory effect on the testes.

Shay et al. have accepted the thesis that testosterone propionate has the dual ability of both inhibiting hypophyseal gonadotropic function and directly stimulating the testes. However, they add an unnecessary and probably incorrect complication by stating that in the first month of life in the male rat "the gonadotropic function [of the hypophysis] is not yet developed." If this were true, there would be no hypophyseal gonadotropic activity to depress and it would be most difficult to explain the testicular depression obtained by administering moderate sized doses of androgens during this period of life.

Phrases such as "testicular stimulation" and "testicular depression" have been used loosely by the present authors and also by others. In the present study these terms have been applied only to testicular weight without regard to the conditions of the germinal epithelium or of the interstitial cells. Actually hypophysectomy results in atrophy of both the seminiferous tubules and interstitial cells. If large enough doses of androgens are given to the hypophysectomized male, the testicular weight is maintained due to maintenance of the seminiferous tubules with their germinal elements, but the interstitial cells remain atrophic (7). When very large amounts of androgen are given to the intact rat the testes remain normal in size and the seminiferous tubules and germinal epithelium remain normal. The interstitial cells, however, become atrophic (9). Small doses given to the intact rat cause decrease in testicular weight together with atrophy of the germinal epithelium and interstitial cells (1–3). Since, under all these conditions, the interstitial cells are atrophic, it is evident that the administered androgen does not have any direct stimulating action on the cells which theoretically produce the endogenous androgen.

The fact that in the intact animal small and large doses of androgen produce diametrically opposite end effects on the seminiferous epithelium leads to some interesting speculation. The evidence shows that the amount of administered androgen necessary to maintain the germinal epithelium is higher than that which inhibits the hypophysis. One might conclude from this that normally the testicular androgen plays no part in the maintenance of the germinal epithelium since the dose necessary to have such an effect would not permit hypophyseal gonadotropic function. This conclusion is not justified. Normally the seminiferous elements are probably exposed to higher concentrations of androgen than is the hypophysis. It is generally assumed that the sex hormones are transported only via the blood stream. This assumption is not

correct. There is ample evidence from experiments involving local application of hormones (14, 15) that sex hormones may be transported to a certain extent by diffusion through tissues. The seminiferous tubules, because of their proximity to the interstitial cells which presumably produce the testicular androgen, may thus be exposed to locally diffused androgen as well as to the blood borne androgens. The hypophysis, on the other hand, is exposed only to the blood borne androgen. We do not wish to be understood as claiming that normally the testicular androgens control spermatogenesis. We only wish to point out that the data obtained from this and similar experiments cannot be used in support of the opposite point of view, i.e., that the testicular androgen normally has nothing to do with maintenance of the germinal epithelium.

We have been criticized (11) for considering 0.1 mg. testosterone propionate daily as a "low" dose in a previous publication relative to this subject (16). This criticism was deserved in that we did not qualify the term. In a prepuberal rat 0.1 mg. is a high dose from a physiological point of view, i.e., it is probably a great deal more than is necessary to maintain the sexual accessories at a normal state in the castrate prepuberal rat. In this present communication, then, the dosages are only considered as large or small in regard to their effect on the germinal epithelium (as reflected in testes weight) and are probably all much greater than the physiological dose. The effects of physiological amounts of androgen are not in any way a subject of this communication.

SUMMARY

Fifteen 31-day-old male rats had been given large doses of testosterone propionate (total 25.0 to 140.0 mg.) starting at 10 days of age. Fifteen untreated litter mates were left in the same cages with the treated animals supposedly serving as controls. The testes of the treated animals were significantly larger than those of their litter-mate controls. After the procedure had been completed it was considered possible that the controls had been contaminated by the androgens given to their heavily treated litter mates. Accordingly a group of normal rats of the same age and weight range were killed. The mean testis weight of these normal animals was not significantly different from that of the treated animals, but was significantly larger than that of the litter-mate controls.

It was concluded that very large doses of androgens neither stimulate nor depress the testes, but maintain them at a normal size.

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ABSORPTION OF PELLETS OF CRYSTALLINE TESTOSTERONE, TESTOSTERONE PROPIONATE, METHYL TESTOSTERONE, PROGESTERONE, DESOXYCORTICOSTERONE AND STILBESTROL IMPLANTED IN THE RAT

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THE ADMINISTRATION of crystalline hormones by the implantation of pellets was first described by Deanesly and Parkes in 1937 (1). Since that date, these workers and others have published numerous observations on the efficiency of this method. The present report is concerned with the relative absorption rates of pellets of some of the crystalline hormones, together with certain supplementary observations. The author hopes to publish at a later date the results of his extension of the investigation to other hormones.

MATERIAL AND METHODS

The author prepared all pellets in a modification of the Hartman apparatus (2), the principle of this ingenious device being retained (fig. 1). The metal parts were were made of brass or steel. The upper (A) and lower (B) plates were carefully lapped. The 7 mm. deep holes in the upper plate were made with a 0.042 inch drill and then reamed so as to flare very slightly downward, as recommended by Hartman. The upper plate was so constructed that additional groups of holes of different diameters can be made at two other points. The rod inserted into the lower end of the ram (C) was made by removing the cutting portion from, and grinding a flat end on a 0.0469 inch drill; this rod fitted snugly into the reamed holes in the upper plate. The tamper (D) was made from an ordinary dissecting needle ground flat at one end.

In practice, the upper and lower plates were bolted together. The crystalline hormone was then packed as firmly as possible into the holes with the aid of the spatula (E) and tamper. When the holes were full, the ram was set up as illustrated (fig. 2) The weight (60 gm.) was raised to the adjustable upper collar and dropped onto the fixed lower collar. In all cases, the upper collar was set at a height of 10 cm. and the weight was dropped 35 times from the full height for each pellet. The arm (fig. 1, F, and fig. 2) supporting the ram kept the latter vertical and prevented possible breaking of the tempered drill rod at the ram's lower end. The standard technic for preparing the pellets ensured each pellet receiving the same amount of compression.

In order to remove the pellets, the upper plate with the ram in position was released from the lower plate and placed on the wooden cup (fig. 1, G). The bottom of the latter had been padded with a layer of cotton batting covered with lens paper. Plate, ram and cup were then placed in the press in such a way that the concave lower end of the hand screw engaged the rounded upper end of the shaft of the ram (fig. 3). Since the press was solidly made of ¼ inch brass, the necessary pressure could be exerted by the hand screw on the ram in order to expel each pellet in turn, the pellets

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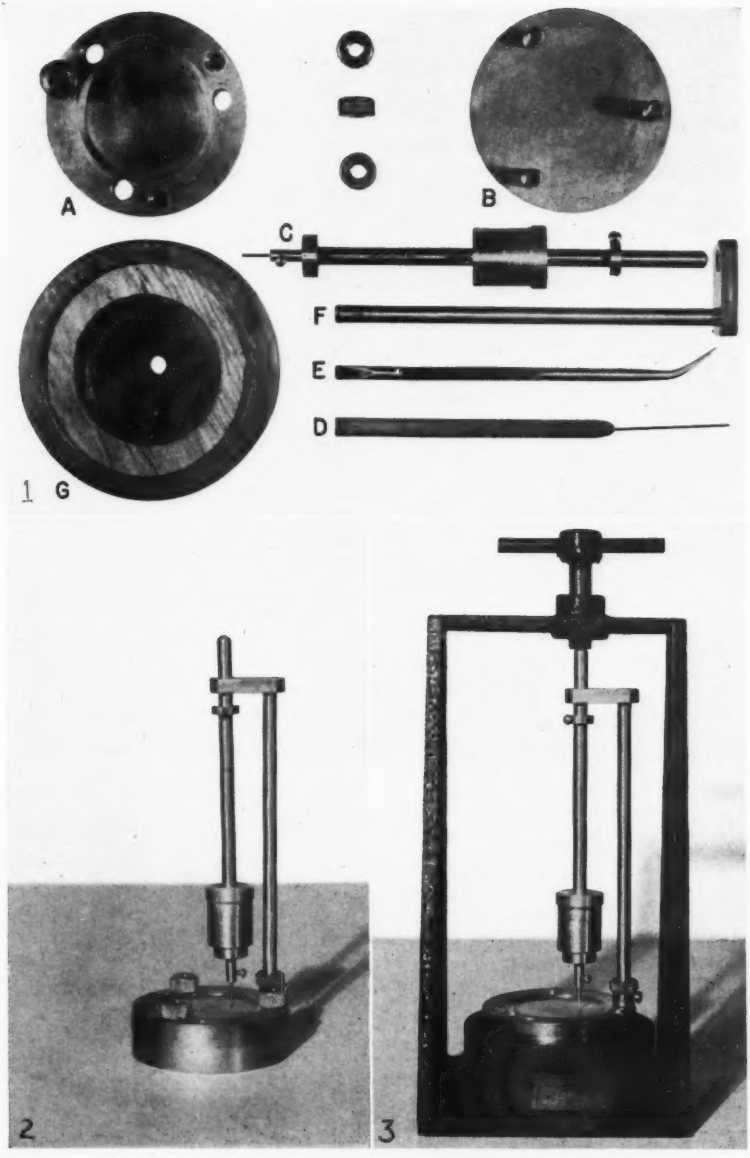

Fig. 1. THE PELLET MAKING APPARATUS DISASSEMBLED, Fig. 2. ASSEMBLED FOR PREPARATION OF THE PELLETS, Fig. 3. PLACED IN THE PRESS FOR REMOVAL OF THE PELLETS. Somewhat reduced.

dropping onto the padding at the bottom of the cup. By turning the handle of the screw slowly and carefully, all pellets could be expelled intact.

Some care was required in the handling of the pellets, since they were made with-

out excipient and broke if roughly handled. After any adherent particles had been removed with a soft brush, the pellets were revealed as smooth, glistening, slightly tapered cylinders. With the technic used, the pellets, which weighed 6–10 mg. each, were found to average 1.6 mg. for each mm. of length. Twenty pellets were made of crystalline testosterone; 16, of testosterone monopropionate; 12, of methyl testosterone; 18, of progesterone; 17, of desoxycorticosterone; 14, of stilbestrol (4:4-dihydroxy-α-β-diethylstilbene).

The rats used were healthy, intact males and females maintained on a balanced and plentiful diet. All animals were sexually mature; nearly all were fully grown; none were senile.

Just prior to implantation, each pellet was weighed carefully on a laboratory

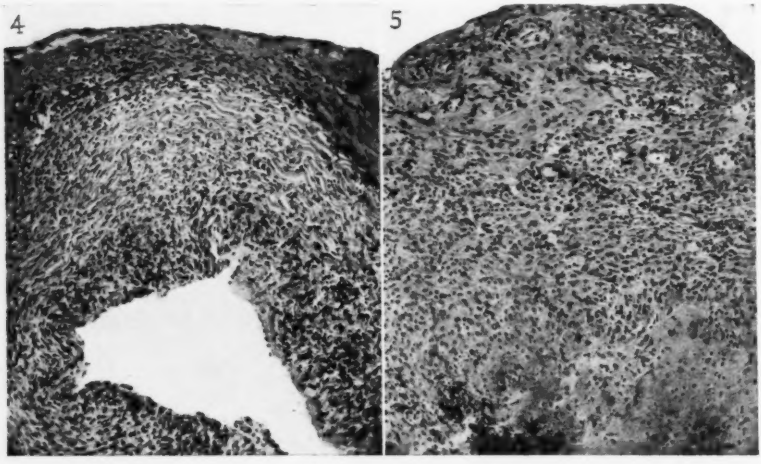

Fig. 4. SECTION THROUGH A TYPICAL CAPSULE (methyl testosterone pellet in situ 29 days). Some fixation shrinkage has occurred. ×105. Fig. 5. SECTION THROUGH A THICK, ATYPICAL CAPSULE (testosterone propionate pellet in situ 10 days). The inner surface of the capsule is at the bottom of the picture. Note (above) blood vessels in outer part of capsule. ×105.

balance accurate to 0.1 mg. Each rat was anesthetized with ether, weighed and marked. One pellet was then implanted subcutaneously in the lower right abdominal quadrant by means of a #13 hypodermic needle, using Hartman's technic (2). Each rat received one pellet only. No pellets were reimplanted as such after recovery although, in some cases, used pellets were thoroughly pulverized and then compressed into new pellets.

At varying intervals after the pellets had been implanted, the rats were killed with ether and weighed. A ventral skin incision was made and the pellet, together with the connective tissue capsule it had acquired, was removed by blunt dissection. The capsule was then cautiously removed and in some cases fixed in Bouin's fluid for sectioning. The fixed capsules were sectioned serially at 10μ and stained with hematoxylin and eosin. Fresh tissue was also teased and stained with methylene blue. The pellet was dried to constant weight in a $CaCl_2$ desiccator and weighed. The percentage reduction in weight of the pellet was then calculated and plotted graphically against the number of days the pellet had been in situ.

RESULTS

Within a week of the time that a pellet was implanted it had developed a transparent but recognizable tissue capsule. As time passed, such a capsule became gradu-

ally tougher, thicker and less transparent. The capsules fitted the pellets closely but did not adhere to them. Histological study of serial sections of the capsules showed that they consisted of circumferentially and very compactly arranged elastic and collagenous fibers interspersed with fibroblasts, lymphocytes, and numerous macrophages (fig. 4). Hartman has commented on the "connective tissue hypertrophy and infiltration of polymorphs" in the capsules he studied (2). Most capsules were smooth on their inner and outer surfaces. Adipose cells were sometimes present at the periphery of a capsule. Small blood vessels, invisible macroscopically, could be recognized in variable numbers in the capsule wall. The structure of the capsules was uniform. No correlation could be made between the histology of the capsule and the nature of the hormone.

When pellets were removed within a few days after implantation there was sometimes evidence of local inflammation. More rarely, numerous lymphocytes and a macroscopically thickened, opaque, and vascular capsule were observed (fig. 5). Pellets having this type of capsule are indicated on the graph by encircled dots. It will be seen that, after the first 10 days following implantation, such pellets had an absorption rate somewhat different from that of the others. In the great majority of cases, however, local reaction to implantation of a pellet was limited to the formation of a normal capsule.

Pellets made from all hormones except desoxycorticosterone were firm and solid upon removal. The pellet surface was only slightly roughened, if at all. The desoxycorticosterone pellets were relatively soft, although not to the point of disintegration. Such softening can be correlated with the fact that desoxycorticosterone pellets were absorbed more rapidly than any others which have been studied.

The absorption data (fig. 6) indicate that the relative periods required under the conditions of the experiment for 90% absorption of the 6 hormones studied are as follows: desoxycorticosterone, 27 days; testosterone, 31 days; methyl testosterone, 36 days; stilbestrol, 51 days; testosterone monopropionate, 61 days; progesterone, 88 days. It should be emphasized that the absolute periods required for the absorption of a given type of pellet could be considerably altered by changes in the shape and hardness of the pellets, in the site of implantation (e.g., intramuscular instead of subcutaneous), and perhaps by other factors as well. A survey of the pertinent literature gives abundant evidence on this point.

The absorption curves were established after the data were plotted graphically. It is interesting to compare these curves with a theoretical curve for the absorption of a cylinder. Such a curve may be obtained as follows: a hypothetical cylinder of the approximate proportions of a pellet is postulated. If it is assumed that the dimensions of such a cylinder are diminishing at a constant rate, the diminution in volume may be computed with the formula $V=\pi\ (r-x)^2\ (h-2x)$, V representing the volume; r, the radius; h, the height; x, the thickness of the layer lost by absorption in a given unit of time. If the successive values for V are plotted against time, the resulting curve is very similar in its shape to the curves actually obtained from the pellet weight data; the shape of the curve up to 90% is indicated exactly by the curve for desoxycorticosterone (fig. 6). Any differences between the actual and hypothetical curves are presumably due to the probability that the pellets were not necessarily absorbed at a constant rate. An interesting feature of the hypothetical curve is that, while it ascends in almost linear fashion to over 90%, it then rapidly becomes less and less steep, so that the amount of time required for absorption of the last 8% is nearly one-third as long as that required for the absorption of the first 92%. Since it was impracticable in most cases to attempt to remove and weigh pellets which were more than 90% absorbed, the absorption curves were determined only to approximately the 90% level.

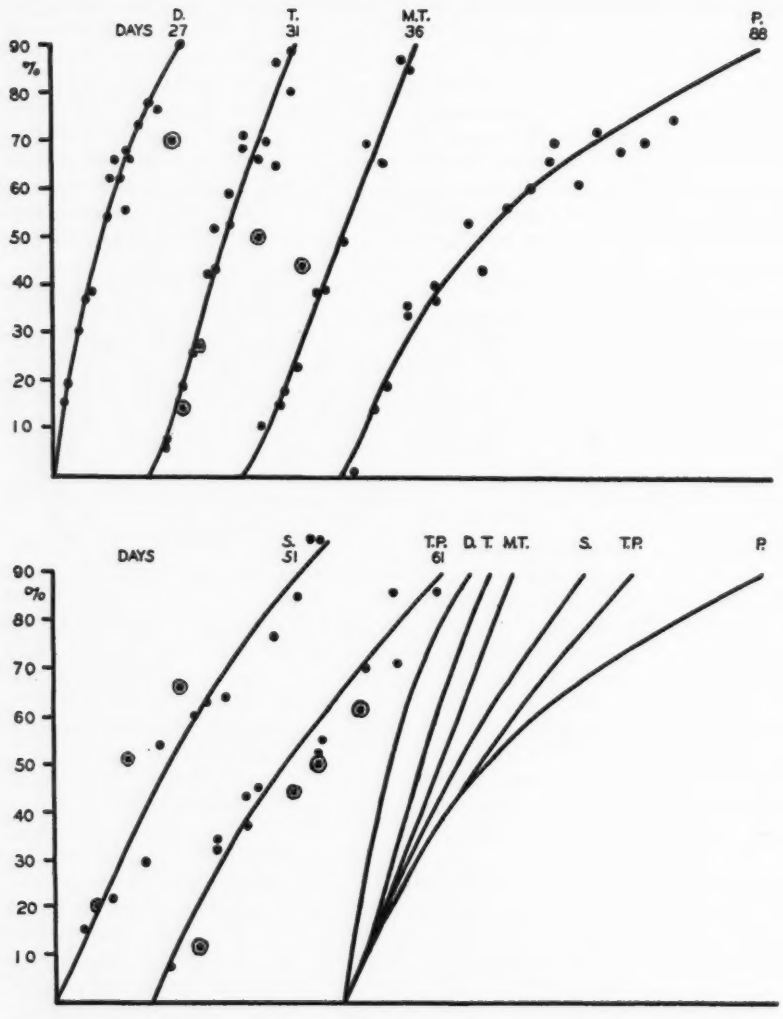

Fig. 6. Absorption curves for desoxycorticosterone (D.), testosterone (T.), methyl testosterone (M.T.), progesterone (P.), stilbestrol (S.), and testosterone propionate (T.P.).

Stilbestrol invariably caused a significant weight loss in the 14 rats to which it was administered; the loss in weight ranged from 0.3 to 3.6 gm. for each day (average) a pellet was in an animal. Weight losses of more than 10 gm. occurred in 20 of the 83 other rats receiving pellets. Nearly all of these weight losses occurred during hot summer weather, and may well have been due to this factor. It would appear, however, that stilbestrol definitely has some toxic effect. One animal was found dead while bearing a methyl testosterone pellet.

The sex of the rats had no constant differential effect on the absorption rates.

DISCUSSION

A variety of factors were involved in the gradual disappearance of the pellets. The basic mechanism appeared to be the dissolving of the pellets by the tissue fluid which moved over their surfaces. The solidity of most of the pellets upon removal indicated that their absorption was chiefly, if not entirely, a surface phenomenon; this conclusion has also been reached by Deanesly and Parkes (3). The possibility exists that the pellets may be partially phagocytized by macrophages, but no evidence for this hypothesis has been secured. The various points on each graph showed, in general, little scattering around the curve. Part of such deviation as did occur was no doubt due to small errors in weighing the pellets. The deviation of other points, however, may well, as suggested by Hartman (2), have been due to slight normal individual variations in the rate at which tissue fluid moved over pellets of a given substance in individual rats. Examination of the graphs shows that the scattering was usually caused by pellets which had been in situ a relatively long time. Most of the comparatively large variations could be correlated with atypical capsules and presumably concomitant alterations in the normal movement of tissue fluid within the capsules; the development of thick, sometimes highly vascular capsules (indicated by encircled dots) was associated with atypical absorption rates of the contained pellets in the 9 cases in which the latter were in situ for 10 days or longer.

The fact that the sex of the rats had no constant differential effect on the absorption rates seems to indicate that absorption of pellets of the types studied was independent of the endogenous sex hormones in the general circulation. Similarly, Deanesly believes that the absorption of estrone and estradiol tablets is independent of the functional requirements of the animals bearing the tablets (4).

The data for testosterone and testosterone propionate confirm Deanesly and Parkes' original observation that pellets of this ester are absorbed less rapidly than the free hormones (1). Experiments now being carried out by the author show that the same generalization may usually be applied to other hormones and their esters.

While the literature contains a number of references to the use of pellets, precise data on absolute, and particularly on relative, absorption rates are limited. For reasons already stated, absolute absorption rates for a given hormone are not directly comparable unless a number of important factors (pellet shape and hardness, implantation site, etc.) are also comparable. For the same reasons, relative absorption rates become significant when they are obtained under uniform conditions. Pertinent data from the literature may well be summarized at this point. It should first be emphasized that, with one exception, previous workers have not stated that the pellets and tablets they used (usually much larger than the author's) were made in a uniform manner for a given study, although such uniformity may sometimes have been maintained, nor in the various mammals used has the implantation site always been specified.

In the pioneer studies it was found, approximately, that in 10 days testosterone tablets were ⅔ to ¾ absorbed and testosterone propionate tablets ⅕ to ¼ absorbed (1, 6). Later, the same workers reported the following percentage absorption of tablets in 30 days; testosterone, 24 to 25%; testosterone propionate, 17%; stilbestrol, 10%; progesterone, 35 to 52.5% (3). If calculations are made from Deanesly's recent data, it appears that testosterone had an average absorption of 21%, and methyl testosterone of 22%, in 30 days (7). Values correspondingly compiled from a study of the absorption of progesterone and desoxycorticosterone indicate that, on a 30-day basis, the average absorption of progesterone was 19.8%, while that of desoxycorticosterone was 66.8% (8). A comparison of small methyl testosterone and testosterone propionate pellets implanted in the spleen revealed that an average of 0.089 mg. of methyl testosterone and 0.065 mg. of testosterone propionate was ab-

sorbed daily (9, 10). A study made with very large androgen pellets indicated that methyl testosterone is absorbed more rapidly than testosterone monopropionate and that testosterone is absorbed more rapidly than methyl testosterone (11, 12).

A very recent series of observations has been made with small testosterone, methyl testosterone, and testosterone propionate pellets which were said to have been uniformly compressed (13). The pellets, removed after 10 to 64% absorption (calculated from authors' data) had occurred, showed absorption rates fairly closely paralleling those observed in the present study.

Several workers have pointed out that the absolute amount absorbed daily from a pellet gradually diminishes (3, 4, 5). The author has been able to supply additional evidence on this point. An interesting feature of the curves in fig. 6, however, is that, with the exception of the curve for progesterone, they are almost linear. This means that an implanted cylindrical pellet of desoxycorticosterone, testosterone, methyl testosterone, testosterone monopropionate, or stilbestrol should supply roughly (although not exactly) the same amount of hormone daily until the pellet is about 90% absorbed. This fact should be of significance in the experimental and clinical use of pellets.

SUMMARY

The apparatus and technic for preparing uniformly compressed crystalline hormone tablets of uniform diameter is described. Twenty testosterone, 16 testosterone monopropionate, 12 methyl testosterone, 18 progesterone, 17 desoxycorticosterone, and 14 stilbestrol (4:4-dihydroxy-α-β-diethylstilbene) pellets were implanted subcutaneously in an equal number of rats. The implanted pellets underwent a nonspecific encapsulation. The pellets, which originally weighed 6 to 10 mg. each, were removed at intervals, and the losses in weight were precisely determined. Graphic analysis of the data showed that, under the conditions of the experiment, 90% absorption of desoxycorticosterone occurred in 27 days; of testosterone, in 31 days; of methyl testosterone, in 36 days; of stilbestrol, in 51 days; of testosterone monopropionate, in 61 days; of progesterone, in 88 days. The significance of the absorption curves is discussed. The sex of the rats had no differential effect on the absorption rates. Stilbestrol invariably caused a significant loss in body weight.

The author wishes to express his appreciation and gratitude to the following persons whose generous cooperation made these experiments possible: the staff of the Edward Martin Biological Laboratory of Swarthmore College, for providing facilities and animals to begin the experiment; Mr. Bruce Valentine, for constructing the pellet apparatus; Dr. Erwin Schwenk of the Schering Corporation, for the crystalline hormones used.

Preliminary reports of these data have appeared elsewhere (*Anat. Rec. Suppl.* 79: 22. 1941; *Science* 93: 404. 1941).

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EFFECT OF TESTOSTERONE PROPIONATE UPON REPRODUCTION IN THE FEMALE

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IN VIEW of the numerous contributions in the literature recommending the administration of androgenic substances for various gynecological disorders, it has seemed advisable to report observations of the effects of male sex hormone upon the reproductive ability of female animals after receiving repeated injections of testosterone propionate. Observations of the fecundity of the progeny of these animals will be reported herewith. A preliminary report concerning this work has been presented in a previous paper (1).

METHOD

Twenty-five healthy adult female white rats, ages 4½ to 6 months, were selected for this study. They were isolated in cool, darkened, well-aired, clean cages and fed a diet of Purina Checkers, lettuce, and beef heart, supplemented by cod-liver oil. Daily vaginal smears were made over a long enough period of time (approximately five weeks) for us to ascertain with certainty that regular estrous cycles occurred. Six of these animals were then segregated for controls.

Each of the remaining 19 female rats were injected daily with 0.2 cc. of sesame oil containing 2 mg. of testosterone propionate.[1] It was observed by means of the vaginal smears that estrogenic activity decreased, and that after the fifth daily injection all of the animals so treated were in diestrus and remained in that state as long as the injections were continued. All of the animals developed hypertrophy of the phallus and most of them became more irritable and pugnacious during the course of the experiment. These phenomena disappeared after the injections ceased.

Four healthy adult male white rats, 6 months of age and of the same Wistar strain as the females used in the experiment, were mated with the controls. Pregnancy developed within 4 weeks and all females had normal litters, demonstrating the fertility of the males to be used in the experiment.

These males were then placed in cages with the females which were receiving daily injections of testosterone propionate. These matings were continued for 4 weeks during which time the injections were continued. At no time was coitus observed nor were copulation plugs seen. At the end of this 4-week period the males were removed and the injections of testosterone propionate which the females had been receiving were discontinued. None of the 19 androgen injected females bore litters despite being caged with fertile males over a 4-week period.

Vaginal smears of the 19 treated rats were observed daily after the discontinuance of the injections of testosterone propionate. Estrous cycles appeared again and as-

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[1] Testosterone propionate, in the form of Perandren, was furnished by the Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

sumed a regularity similar to that noted prior to the injections of male sex hormone. Estrus recurred in most of the animals between the fifth and the eighth day after the last injection of testosterone propionate. One female rat showed large cornified vaginal cells in the vaginal smear within 48 hours after receiving the last androgen; two remained in diestrus for 14 days.

The androgen injected females were observed over a period of 30 days after the injections were discontinued and the males withdrawn. During this time, in addition to the recurrence of regular estrous cycles, the phallic hypertrophy disappeared and the behavior of the females returned to normal. The males previously described were again caged with this group of female rats. Litters were born to 18 of the 19 female animals within 38 days after they had been reunited with the males. Two of the females destroyed some of their young. One of these litters was apparently born prematurely. The average litter consisted of 7.3 offspring. These items are in proportion with the remainder of our colony, except that our average normal litter consists of 7.1 offspring.

The progeny of the androgen treated females appeared grossly normal. They suckled, developed hair, and foraged for themselves in a normal manner. The females covered their young and permitted them to suckle in a fashion similar to that of the controls in all respects.

The offspring had grossly normal genitalia both externally and internally. It was deemed advisable, however, to study their reproductive powers, since insofar as could be ascertained no one has ever determined what, if any, effect the administration of an androgen to a female prior to her pregnancy would have upon the genitalia of her subsequent progeny. Therefore, when these litters were 4½ months old, 10 females and 10 males born of female rats which had received daily injections of testosterone propionate prior to conceiving were caged together. Normal litters were obtained without delay in every case. Since the original observation on this group of the progeny of the androgen injected females, the remainder of the offspring have either been mated together or with the offspring of non-injected mothers. Their reproductive records equal those of the rest of our colony. Within recent months, the offspring of this second generation has reached maturity and are likewise reproducing satisfactorily.

DISCUSSION

Administration of androgenic substances for various gynecological disorders, especially functional uterine bleeding, dysmenorrhea, and mastopathies, has been widely recommended. The commonly utilized androgen has been testosterone propionate, a synthetic male hormone of great potency. Injections of this androgen are known to inhibit cyclic phenomena in the female genitalia of laboratory animals and of human beings. When given therapeutically, it appears that the androgens are effective in most instances and that their use is worthwhile, provided no injurious manifestations develop. When, however, a substance is so potent that it completely inhibits the estrous cycles of animals and prevents menstruation in human beings, then its use must be recommended only after considerable investigation has been made of its immediate and possible future effects upon the female organism.

Studies in this laboratory and elsewhere have pointed out the facts that the androgenic effect of the male sex hormone on the female genitalia is secondary to pituitary changes, and this effect is maintained only a short time after the injections are discontinued. Insofar as could be determined no one had applied the critical test of attempted reproduction following prolonged androgenic therapy although Scipiades (2) noted that injections of testosterone do not necessarily render experimental ani-

mals sterile. This factor of possible change in fecundity becomes a vital one when its remembered that most of the patients receiving androgenic therapy for functional uterine bleeding and dysmenorrhea are in the childbearing years. It is imperative that data be available from which conclusions can be drawn relative to the possible effect of treatment on the future gestations of these women. Greene and Ivy (3) and others have demonstrated gross deformities in the genitalia of rats born of mothers that received male hormone during their pregnancies. Even a remote implication that any similar effect might be produced in human gestations as a result of the use of androgenic substances, administered therapeutically at any time prior to conception, would be a strong contraindication to their use. Mazer and Mazer (4) reported that 4 of their group of 38 women who had received androgenic therapy subsequently developed normal gestations. The work herewith reported bears out the conclusion that androgenic therapy, administered prior to pregnancy, has no effect on fertility of the injected animal nor upon the fecundity of the offspring.

SUMMARY

No litters were born to 19 female rats which received 2 mg. of testosterone propionate each daily despite their being caged with known fertile males. Estrus ceased during the administration of the androgen and all of the female animals developed clitoridal hypertrophy. Following cessation of the injections estrus returned to normal and, after again caging with the same males as used before, healthy litters were born. These progeny later also bore normal offspring.

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THE BENEFICIAL ACTION OF TESTOSTERONE IN EXPERIMENTAL RENAL ATROPHY CAUSED BY LIGATURE OF THE URETER

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AFTER IT HAD BEEN SHOWN that various steroid hormones, especially the androgens, exert a definite "renotropic" action inasmuch as they cause an enlargement of the kidney tubules (1, 2, 3), it became of interest to establish whether this renotropic effect could be used pharmacologically in cases in which a stimulation of the renal tissue is desirable. Animal experiments showed that testosterone exerts a protective action against the kidney damaging effect of sublimate (4) and that the compensatory renal hypertrophy following unilateral nephrectomy is considerably increased by testosterone propionate treatment (5).

In continuation of these investigations the question arose whether the renal atrophy, which is normally caused by ligature of one ureter, could be delayed by androgen treatment. This problem appeared of some practical importance inasmuch as we felt that it might lead to an interesting clinical application of the renotropic steroids in the comparatively frequent condition of hydronephrosis with subsequent renal atrophy.

In a group of 20 female albino mice weighing 19–23 gm. (average 21 gm.) the left ureter was ligated very close to the renal pelvis. After the operation the animals were subdivided into two groups. Ten mice received 20 daily subcutaneous injections of 4 mg. of non-esterified testosterone in 0.1 cc. of peanut oil, while the other 10 served as non-hormone-treated controls. On the day following the last injection all animals were killed and the kidneys fixed for histological examination. It was noted at autopsy that even upon naked eye inspection the hydronephrotic kidneys of the testosterone treated animals appeared in somewhat better condition and more reddish in color than those of the controls. This difference became much more obvious upon histological examination. Although a pronounced degree of hydronephrosis was evident both in the treated and in the control animals, the renal structure remained much better preserved in the mice receiving testosterone than in the controls. In the latter only a few atrophic tubules and glomeruli were visible and in the great majority of the cases most of the renal remnant was substituted by proliferating connective tissue and round cell infiltrations. In some cases most of the kidney remnant became necrotic and in certain areas actual pus formation was evident. The hydronephrotic kidneys of the treated animals, on the other hand, were evidently much better preserved, in fact in certain areas the convoluted tubules showed obvious signs of hypertrophy (fig. 1, 2).

These experiments indicate that an androgen such as testosterone can actively delay the kidney involution which usually follows during the development of hydronephrosis subsequent to occlusion of the ureter. It must be emphasized that in our experiments we used very large doses of testosterone and up to the present our work

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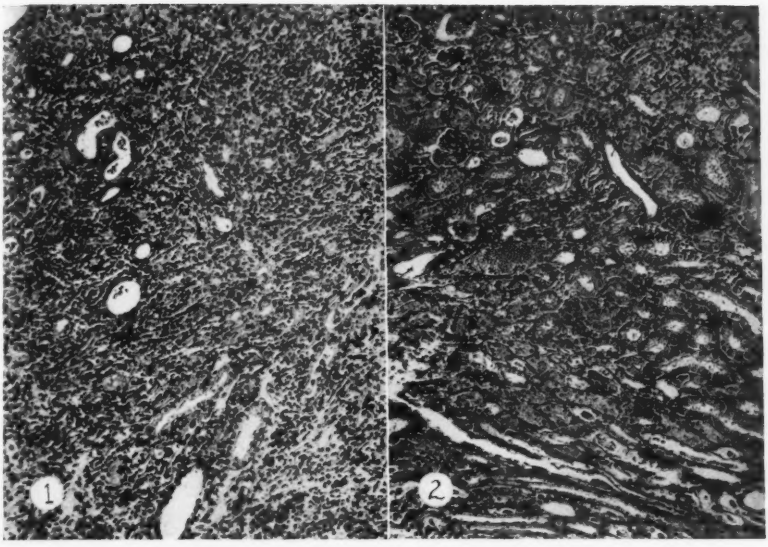

Fig. 1. KIDNEY FOLLOWING LIGATION OF THE URETER WITHOUT ANY HORMONE TREATMENT. Note the almost complete disappearance of renal tubules and glomeruli. The few atrophic tubular remnants are surrounded by proliferating connective tissue and round cell infiltrations. Fig. 2. KIDNEY FOLLOWING LIGATION OF THE URETER IN A TESTOSTERONE TREATED ANIMAL. Note the excellent preservation of the convoluted tubules most of which are actually hypertrophied. In spite of the ureter occlusion, the Bowman's capsules show the hypertrophy characteristic of testosterone treatment.

was limited to the mouse, so that further experiments will be required to establish whether these findings are applicable in clinical cases of hydronephrosis.

SUMMARY

Experiments on the mouse indicate that the renal atrophy which usually develops subsequent to the hydronephrosis caused by unilateral ligature of the ureter may be inhibited and delayed by testosterone administration.

The expenses of this investigation were defrayed by a grant received from the Committee on Research in Endocrinology, National Research Council. The authors are especially indebted to Drs. Gregory Stragnell and Erwin Schwenk of the Schering Corporation of Bloomfield, New Jersey for the testosterone used in these experiments.

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RADIOACTIVE IODINE AS AN INDICATOR IN THYROID PHYSIOLOGY

III. IODINE COLLECTION AS A CRITERION OF THYROID FUNCTION IN RABBITS INJECTED WITH THYROTROPIC HORMONE[1]

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IN PREVIOUS PUBLICATIONS (1, 2) we have discussed the collection of iodine by the thyroid in short intervals after injection. The experiments described covered both normal and hyperplastic glands. The thyroid iodine collection was referred to the size and gross appearance of the gland, and to the method of treatment of the animal. Those experiments, while quantitative, involved parameters which were known not to be uniquely or specifically indicative of thyroid function. The data served, however, to establish approximately the iodine collection by the gland in various functional states. The present experiments were designed to correlate the iodine collection with known criteria of thyroid function, in particular the basal metabolic rate and the mean acinar cell height. This paper will be concerned with rabbits injected with anterior pituitary thyrotropic hormone.

PROCEDURES

The standard dose of thyrotropic hormone was nominally 15 guinea pig units of anterior pituitary thyrotropic hormone,[2] administered subcutaneously every other day during the period of preparation of the animals.

An intravenous dosage of 5 mg. of iodine (labelled with the 25-minute isotope I^{128}) per standard 2 kg. animal was used in all experiments. In order to study the effect of pretreatment with iodine on the collection of a 5 mg. dose, various pretreatment dosages were used. In some experiments iodine labelled with radioactive isotopes other than the 25-minute isotope was used for pretreatment. Adult male rabbits were used exclusively. Each animal was killed 15 minutes after the injection of the labelled 5 mg. dose. As each animal was killed, one lobe of the thyroid was taken for measurement of the iodine collection, and the other lobe was taken for histological study. (Several control experiments showed no significant difference between the iodine collections per unit mass of the two lobes of a thyroid.) Fixation was by 60% alcohol, and hemotoxylin-eosin staining was used. Cell heights were measured by means of an ocular micrometer to the nearest 0.5μ. In order to eliminate personal error as far as possible, all cell heights were measured by the same observer. An average of about 200 cell heights were measured for each gland, these measurements being taken on at least 5 different sections, not more than three cells in one acinus being counted.

Basal metabolic rate procedures. Basal metabolic rates were determined from oxy-

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[1] This research was supported by grants from the Milton Fund and the H. N. C. Gift for Medical and Surgical Research of the Harvard Medical School, and principally by a grant from the John and Mary R. Markle Foundation.
[2] Antuitrin-T, Parke, Davis & Co.

gen consumption in a closed system with the rabbit at 28.5°C. Each metabolic rate determination lasted from 40 minutes to an hour. A satisfactory test was considered to be one in which basal conditions were maintained for at least 10 minutes. At least 3 such tests, taken on different days and agreeing within 10%, were used to establish levels. (This procedure could not be used, of course, when the metabolic rate was changing.) Measured rates were expressed in calories per kg. per hour, no correction for surface area being applied. The normal level was determined for each animal before any experiments were undertaken. The effects of various kinds of treatment were expressed in terms of percentage deviation from the normal level thus established for each animal.

The technic of radioactive iodine measurements has been described previously (1). Quantitative indices were constructed as follows.

1. Size of thyroid relative to body weight—Thyroid Index

$$TI = \frac{\text{Thyroid Weight in mg.}}{\text{Body Wt. in kg.} \times 10}$$

2. Collection of iodine by the thyroid—Thyroid Concentration Coefficient

$$TCC = \frac{\text{Amount of I collected by thyroid}}{\text{Amount expected from uniform diffusion throughout the body mass}}$$

3. Basal metabolic rate
4. Cell height—CH = average acinar cell height in microns

RESULTS

Table 1 shows the result of a correlation analysis (3) of the five variables of the experiments: TI, TCC, CH, B.M.R., and time of treatment. This table is for data on 9 animals receiving thyrotropic treatment only. The analysis gives the linear correla-

TABLE 1. RANK CORRELATION COEFFICIENTS FOR THYROTROPIC HORMONE TREATED ANIMALS DURING PERIOD OF ADMINISTRATION

Variables	Correlation coefficient	P
Last B.M.R.—Time	+.08	
Last B.M.R.—CH	−.32	
Last B.M.R.—TI	+.08	
Last B.M.R.—TCC	+.12	
CH—Time	−.38	
CH—TI	+.23	
CH—TCC	−.56	.12
TI—Time	−.58	.10
TI—TCC	−.80	.01
TCC—Time	+.40	

Time refers to the duration of treatment with thyrotropic hormone.

'P' is the probability that a correlation is due to a chance variation. The probability that a correlation is statistically significant is 1−P.

Average normal values for untreated animals: TI, 7.5; TCC, 15.0; CH, 2.18μ; B.M.R. zero by definition.

tion coefficient taken on the basis of rank. The results of this correlation study were used to draw the curves in the graphs showing the relations between the variables. It should be remembered that this analysis gives only linear correlation, and a low absolute value of correlation thus indicates either no correlation or a non-linear relation.

Variations of B.M.R., TI, CH, and TCC with time of injection with thyrotropic hormone and with time of subsequent treatment are shown in figure 1. Subsequent treatment includes either rest, iodine or both. The TI and CH reach a maximum in a few days and decline together thereafter. The B.M.R. reaches its maximum somewhat

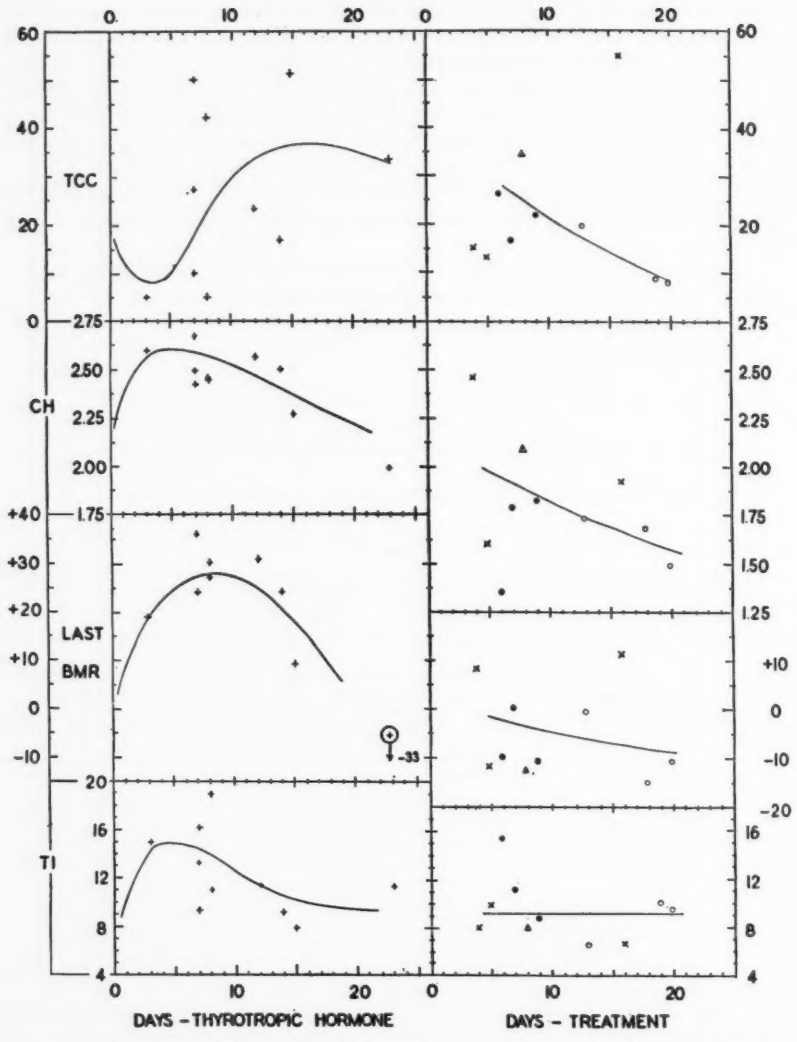
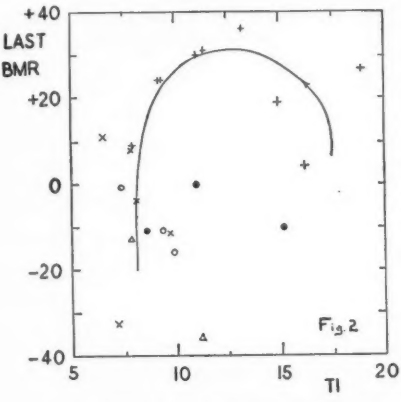


Fig. 1. VARIATIONS OF B.M.R., TI, CH AND TCC WITH INJECTED TIMES. + Treatment with thyrotropic hormone; △ Thyrotropic hormone and rest; ○ Thyrotropic hormone, iodine and rest; ● Thyrotropic hormone and iodine; × Multiple labeling.

later and then declines. The TCC drops slightly at first and then increases as the other variables decrease. The behavior after 14 days is somewhat uncertain. We interpret these data as follows. The initial decrease in TCC is indicative of increased threshold for iodine collection in large hyperplastic glands. The subsequent increase in collection as the gland diminishes in size can be attributed to a decreasing threshold. All parameters are decreased by rest or iodine treatment subsequent to thyrotropic hormone.

The interrelations of the various criteria of thyroid function are given in figures 2 to 7. The curves are drawn and arrows indicated on the basis of the correlation analysis. The arrows show the direction of change with time of treatment.

The most significant linear correlation between time of administration of thyrotropic hormone and any other variable is (−.58) for the linear correlation between time and thyroid index (which has a 90% chance of being statistically significant). The negative value indicates that the thyroid index decreases with time. Since the normal thyroid index is about 7.5, reference to figure 1 shows that the thyroid index must



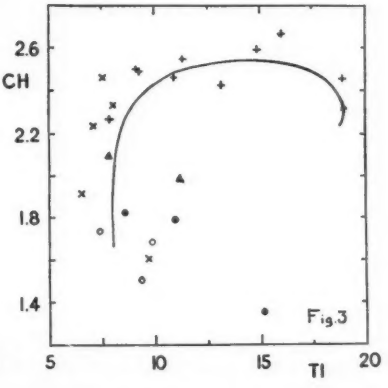


Fig. 2. LAST B.M.R. VS. THYROID INDEX. The last B.M.R. of animals injected only with thyrotropic hormone rises to a maximum of about +30 at thyroid index about 12, and reaches lower levels at lower thyroid indices. The B.M.R. after treatment with iodine or after cessation of thyrotropic hormone administration is below normal, and shows no pronounced variation with thyroid index. Since even the last B.M.R. lags behind the functional state of the thyroid by several days, we interpret the data as showing that the functional activity of the gland is at a maximum under thyrotropic stimulation at a thyroid index greater than 12, and is markedly decreased by iodine treatment. A plot of average B.M.R. vs. thryoid index follows essentially the same curve.

Fig. 3. VARIATION OF CELL HEIGHT WITH THYROID INDEX. Animals receiving only thyrotropic hormone are found to have both increased cell height and larger glands (higher thyroid index). Animals receiving treatment with iodine in addition to thyrotropic hormone, or in which the hormone treatment is discontinued, or both, show a decrease in cell height to below normal levels. We interpret this as showing an increase in the amount of acinar colloid relative to total weight.

at first rise sharply and then gradually decrease as the administration of thyrotropic hormone is continued.

The TCC is linearly correlated with thyroid index (−.80) (chance of statistical significance .99). The inverse relationship (fig. 4) thus shows that the TCC at first decreases, then increases, with time. The inference drawn from this is that the gland, at first much increased in size, initially fails to collect iodine from a 5 mg. injection (within 15 minutes) (fig. 1), but that as it decreases in size, it becomes more and more iodine-hungry, so that the TCC increases to values above normal. The failure to collect I within 15 minutes does not imply that collection will not occur in longer periods of time.

The only other possibly significant linear correlation is that between TCC and CH (−.56) (chance of significance .88). The cell height varies inversely as the TCC, thus roughly parallel to the TI (fig. 3). We thus expect an initial increase in cell height, followed by a subsequent drop. The curve is so drawn in figure 1.

Having thus obtained the variation of TI, TCC, and CH with time, the variation

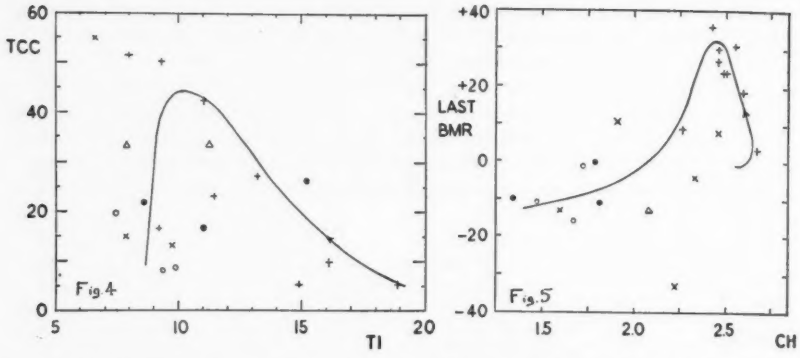


Fig. 4. THYROID COLLECTION COEFFICIENT VS. THYROID INDEX. The largest glands show the smallest iodine collection per mg. of thyroid. As the gland increases in size at first the iodine collection decreases. Then, with the subsequent decrease in size, the iodine collection increases. When iodine is administered the collection of subsequent doses is decreased. The shape of the curve is similar if one plots total iodine collection in place of TCC.

Fig. 5. VARIATION OF LAST B.M.R. WITH CELL HEIGHT. In thyrotropic hormone treated animals, the cell height at first increases markedly, then gradually drops to below normal (2.18μ). The B.M.R. rises, reaching a maximum and then drops below normal. As the B.M.R. drops the cell height drops markedly with treatment.

of B.M.R. with time must be found. The low linear correlation indicates either lack of relation or non-linear relation. The latter is indicated by the experimental points of figure 1.

A comparison of the data plotted in figure 3 with that previously published (2), shows that the data for the present group of thyrotropic hormone injected rabbits fall closer to the data for the animals previously classified as *type 2* than they do to the data for *type 1*. This bears out the suggestion made in that paper that the two types are not sharply distinguished, since these animals were treated in the same way as the

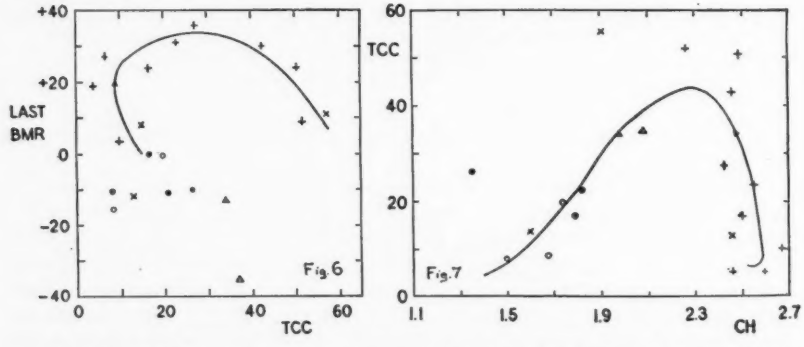


Fig. 6. LAST B.M.R. VS. THYROID COLLECTION COEFFICIENT. As the B.M.R. increases, the TCC at first decreases, then increases. Maximum B.M.R. is correlated with medium TCC, and the lower B.M.R.'s encountered in animals with glands diminishing in size are associated with high TCC. Iodine treated animals show low TCC and low B.M.R. The last B.M.R. is used rather than the average, to diminish the lag in B.M.R. in an animal whose thyroid function is changing.

Fig. 7. THYROID COLLECTION COEFFICIENT VS. CELL HEIGHT. As the cell height decreases from the initially high values, the TCC is at first low and then increases, reaching high values for the animals with CH slightly above normal. Animals treated with iodine show lowest cell heights correlated with lowest TCC.

animals previously listed as *type 1*. The distinguishing characteristic of various animals should probably be the iodine uptake at any given dosage level rather than type classification.

Multiple Labelling Experiments

In 4 thyrotropic hormone injected animals which received labelled doses of iodine of less than 1 mg., no threshold for iodine collection over a period of several days was observed. The time of collection was between 4 and 9 days. In all these animals relatively large collection (17–32γ) was observed. The collection of a second labelled dose in 15 minutes was small. In one animal receiving 25 mg. of iodine 4 days before administration of the first labelled dose, the collection from a 1 mg. labelled dose given after 11 days was only 4γ.

DISCUSSION

Our present picture of the behavior of the thyroid under continued thyrotropic hormone stimulation is somewhat as follows.

As thyrotropic hormone is administered, the TI and CH show an immediate and rapid increase at first. The B.M.R. also increases, but more slowly. The TCC shows an initial drop, indicating that the very large thyroids with large cells have little iodine collecting power for a 5 mg. dose in 15 minutes. After the initial rise, the TI and CH begin to fall slowly. The TCC begins to rise to levels above normal, indicating that the gland is developing iodine hunger. The B.M.R. continues to rise, reaching a maximum at about 8 days. After the B.M.R. begins to drop, the TCC still increases somewhat until about 12 days, after which it probably stops increasing, or perhaps even falls; the data here are insufficient to tell. The iodine collection of animals after 3 weeks of thyrotropic hormone treatment is still above normal.

If thyrotropic hormone injections are stopped (rest), the TI, CH and B.M.R. drop. *The TCC remains high, even when the other measures have dropped below normal, if no iodine is given.* If iodine is given (or iodine plus rest), the TI, CH, TCC, and B.M.R. all drop. Thus the gland becomes involuted. The B.M.R. and CH approach subnormal levels, and the TCC becomes subnormal with sufficient iodine treatment. The size of the gland approaches the normal in either case.

A distinct parallelism may be noted between the behavior of the thyroid under thyrotropic stimulation and the behavior of the islets of Langerhans under anterior pituitary stimulation, as described by Young (4) and by Campbell and Best (5). These authors demonstrated a functional depression in the production of insulin under prolonged anterior pituitary stimulation. The picture given above conforms with that of Hertz and Kranes (6) of functional exhaustion of the thyroid after protracted injections with thyrotropic hormone.

SUMMARY

Using radioactive iodine as an indicator, we have compared the following criteria of thyroid function in rabbits injected with thyrotropic hormone: thyroid iodine collection, acinar cell height, relative thyroid size, and basal metabolic rate. The results indicate that an approximately parallel variation of cell height, basal metabolic rate, and thyroid size takes place. The thyroid iodine collection varies with the other criteria, but not in a parallel fashion. The B.M.R., cell height, and thyroid size show initial rises to a maximum with subsequent decline, which is accelerated by the administration of iodine. All except the thyroid size reach final values below normal when iodine is administered. Thus the result of thyrotropic hormone injections may be regarded as an initial stimulation followed by (*a*) involution if iodine is administered, (*b*) functional depression after prolonged thyrotropic stimulation. The data

accord with the conception of a varying ability to collect iodine in various stages of stimulation, with the higher thresholds corresponding to greater degrees of stimulation, and decreasing threshold during conditions of involution and functional depression.

We wish to express our thanks to Professor R. D. Evans of the Massachusetts Institute of Technology and to Dr. J. H. Means of the Massachusetts General Hospital and the Harvard Medical School for their generous interest and encouragement. The experiments with the long-period isotopes of iodine were expedited by the generosity of Dean L. A. Dubridge of the University of Rochester, and Professor Kenneth L. Bainbridge and Dr. B. R. Curtis of Harvard University. We are glad to acknowledge the technical assistance of Miss Phyllis Brown.

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EFFECT OF TESTOSTERONE PROPIONATE ON CREATINURIA OF EXPERIMENTAL HYPERTHYROIDISM IN MALE AND FEMALE MONKEYS[1]

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CREATINURIA is known to occur in spontaneous and experimental hyperthyroidism in both animals and man (1, 2, 3). Palmer, Carson and Sloan (4) and Kepler and Boothby (5), have used Lugol's solution in reducing the spontaneous creatinuria occurring in exophthalmic goiter and toxic adenoma of the thyroid.

The administration of testosterone propionate[2] reduces the creatinuria which occurs in the eunuchoid (6) and in the rat as a result of creatine feeding (7). Immature and castrate male monkeys exhibit a creatinuria which can be abolished by testosterone propionate. The capacity to retain exogenous creatine is also enhanced by androgen treatment (8).

Since androgens reduce or abolish creatinuria in eunuchoids and castrates, it was of interest to determine the relation of androgens to the creatinuria induced by thyroxin.

METHODS AND MATERIALS

Three male (2 castrate and 1 normal) and 3 spayed female rhesus monkeys (*Macacus mulatta*) were used in this study. The general procedure followed has been described previously (8).

The animals were usually weighed daily before feeding. Two monkeys were injected with 1.0 mg. thyroxin daily; however, when it was felt that they were losing too much weight the dosage was dropped to 0.75 mg., but finally raised to 1.0 mg. again. The other 4 monkeys were given a daily dose of 1.0 mg. thyroxin throughout the course of the experiment.

The experiments were carried out in the following manner: 2 monkeys (591 and 631) were injected with thyroxin for 5 consecutive days after a control period. This was followed immediately by the simultaneous administration of 10 mg. testosterone propionate and thyroxin for 8 days. Three monkeys (601, 613 and 649) were injected daily with 1.0 mg. thyroxin daily, until an intense creatinuria developed (6–7 days), and then for 8 days with 10 mg. testosterone propionate and thyroxin. Following this period, the androgen was withheld but the thyroxin was continued. One monkey (614) served as a thyroxin control. It was injected with 1.0 mg. daily for 11 days.

RESULTS

During the control period, all the monkeys except 1 (601) exhibited a creatinuria which averaged 10 to 15% of the creatinine values. The administration of thyroxin

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[1] Aided by a grant administered by Dr. P. E. Smith from the Rockefeller Foundation.

[2] Testosterone propionate, in the form of Perandren, was supplied through the courtesy of Mr. R. C. Mautner of Ciba Pharmaceutical Products, Inc.

resulted in an intense creatinuria, which manifested itself within 24 to 72 hours after the commencement of treatment. The weight of the animals began to decline with the onset of treatment.

In the castrate or immature male monkey (on a creatine-free diet) creatine rarely exceeds 20% of the creatinine values (8). After the administration of thyroxin, however, the output of creatine may approximate or even exceed the amount of creatinine excreted. The creatine values vary greatly but once they reach a maximum, they invariably exceed 100 mg. per 24 hours.

When the creatinuria became intense (after 5–7 days), androgen treatment to-

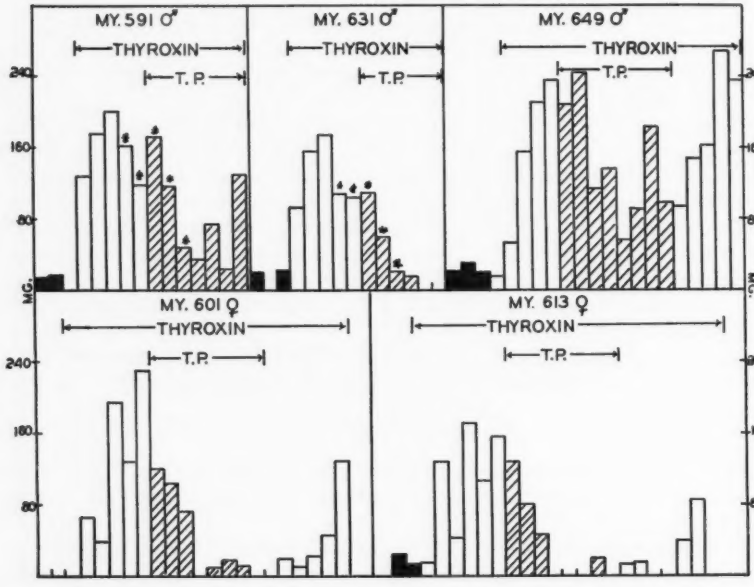

Fig. 1. Daily excretion of creatine (expressed in mg.) for 5 monkeys. Control period, blackened bars; thyroxin period, blank bars; thyroxin and testosterone propionate, cross-hatched bars; thyroxin, 1 mg./day; T.P., testosterone propionate, 10 mg./day; *0.75 mg. thyroxin.

gether with the continued thyroxin was instituted. Within 48 to 72 hours, there is a noticeable decrease in creatine excretion. In 3 of the monkeys, the urine became creatine-free for a few days; when present, it usually was 20% of the creatinine values. In the other 2 animals, the creatinuria was never completely abolished by androgen treatment; however, it fell 30% in one case and 55% in the other. The latter 2 animals showed the greatest creatinuria and largest fall in weight during the thyroxin period. In My. 591, the creatinuria rose to 126 mg. on the last day in spite of the continued androgen treatment. Although the androgen suppresses the creatinuria in some animals it does not prevent any further fall in weight due to the continued thyroxin treatment.

In 3 animals, administration of thyroxin was continued after the cessation of androgen treatment. In all 3, there was a gradual upswing in the creatine excretion toward the pre-androgen levels. However, they were never fully attained in 2.

The figure shows the daily creatine values of the monkeys studied.

The thyroxin injected control showed an intense creatinuria throughout the 11-day period of administration.

There was no significant change in creatinine excretion.

DISCUSSION

Administration of thyroxin to monkeys results in an intense creatinuria. Androgen treatment greatly suppresses or may even abolish this thyroxin-induced creatinuria in both male and female monkeys in spite of the continued thyroxin treatment. Kenyon et al. (6) and Jailer (8) have previously shown that testosterone propionate will reduce or abolish creatinuria occurring in the eunuchoid or in the castrate or immature monkey. In the rat androgen will reduce the creatinuria induced by creatine feeding; however, it cannot hold the creatinuria in abeyance for more than about 10 days (7). The same may hold for the monkey. The experiments reported here were of shorter duration and in 1 animal the creatinuria rose precipitously on the last day of androgen treatment.

Carson (9) and Wilkins and Fleischmann (3) have reported that a thyroid induced creatinuria in man, cannot be maintained unless thyrotoxicosis develops. However, in the monkey 1.0 mg. thyroxin per day is sufficient to maintain an intense creatinuria for the duration of the injection period (11 days).

The decrease in creatine excretion after Lugol's solution in hyperthyroidism is attributed to an improvement in the health of the patient and not to a specific effect (4, 5).

The mechanism of creatine-creatinine metabolism is not clearly understood at the present time. It is thought that experiments with the endocrines may cast some light on the subject. In a recent series of papers, Beard and his collaborators (10–13), reported an induced creatinuria in rats after the administration of gonadotropic extracts, androgen, estrogen, cortical extract, progesterone and even distilled water and saline. They postulate that the creatine-creatinine conversion is dependent upon water metabolism. The hydrolysis of creatinine results in creatine, and since the sex sterols and cortin affect water metabolism, they cause a creatinuria. Their hypothesis that creatinine is converted to creatine has been criticized by Bloch and Schoenheimer (14). It is difficult to see how the injection of but 2 cc. of water into a 200 gm. rat will lead to a creatinuria. The same effect should result from the ingestion of large quantities of water. In the monkey, 20 to 40 cc. of saline injected intraperitoneally has no effect on creatine excretion. While water intake in this group of monkeys was not carefully checked, differences from day to day did occur because the urinary volume varied from 75 to 400 cc., and yet there was no corresponding fluctuation in the creatine values during a particular procedure of the experiment.

If anterior pituitary extracts and sex sterols cause an intense creatinuria, Beard does not explain the fact that in adults where there are circulating hormones, there is no creatinuria; while in children, where there is no physiological evidence for their presence, there is a creatinuria. Creatinuria also occurs in eunuchs and eunuchoids.

SUMMARY

Thyroxin administration to monkeys results in an intense creatinuria. This induced creatinuria was suppressed in 2 instances and abolished in 3 instances by the administration of testosterone propionate. The effect is similar regardless of the sex of the animals.

I wish to express my gratitude to Dr. E. T. Engle for his advice and support of these studies.

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EFFECT OF ADMINISTERING THYROTROPIC HORMONE WITH AND WITHOUT IODINE ON THYROID TISSUE METABOLISM

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THE ACTION of the thyrotropic hormone of the anterior pituitary gland is directly on the thyroid gland (1). That the thyrotropic hormone causes a marked increase in the metabolism of intact animals has been well established (2, 3). This increase in metabolism does not take place in thyroidectomized animals (4, 5). The thyrotropic hormone causes thyroid hypertrophy and evidence of marked stimulation histologically (6, 7, 8). Increased in vitro metabolism of thyroid tissue after in vivo or in vitro stimulation with thyrotropic hormone has been reported (9, 10). Animals treated with thyrotropic hormone and iodine simultaneously show a lessened total metabolic response and less evidence of thyroid stimulation histologically than animals treated with thyrotropic hormone alone (11–14).

In our experiments, we have injected guinea pigs with thyrotropic hormone and potassium iodide, and have determined the metabolic response of the thyroid tissue by in vitro measurement of its oxygen consumption.

METHODS

Female guinea pigs (225–325 gm.) were injected daily for six days with 0.1 cc. subcutaneously of a commerical preparation of thyrotropic hormone.[1] Except for one group killed 12 hours after one injection, animals were killed 24 hours after the last injection. One lobe of the thyroid gland was used for the determination of its oxygen consumption, one lobe for determination of certain chemical constituents.[2] Both lobes were weighed. Some of the glands were used for histological studies. Parallel studies were made on animals receiving a similar amount of the thyrotropic hormone plus 200 mg. per kg. of KI per day injected intraperitoneally.

Oxygen consumption of the glands was determined in a phosphate buffered Ringer's solution containing glucose by the Warburg manometric procedure (15). After killing the animals by a blow on the head, the thyroids were quickly dissected free, sliced and placed in conical Warburg vessels containing 0.2 cc. of 20% KOH in the inner cup. All operations were carried out in a 38° constant temperature room. Measurement of the respiration was begun within 30 minutes after the death of the animal. The oxygen consumption was calculated in the usual manner, QO_2 = cmm. oxygen consumed per mg. dry weight of tissue per hour.

RESULTS

The individual determinations for each day have been plotted in figure 1. The solid line is drawn through the average values for each day following the injection of thyrotropic hormone only; the dotted line represents the average data when KI was

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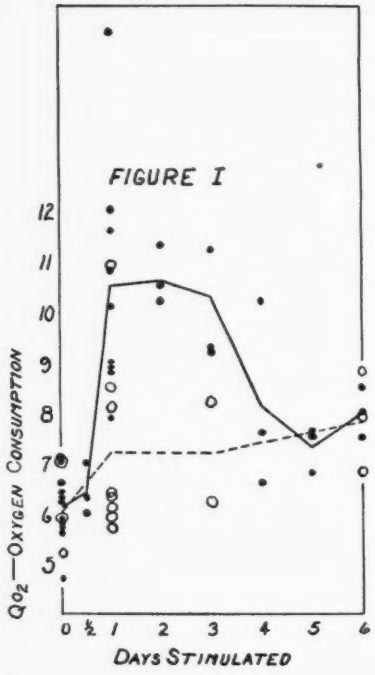
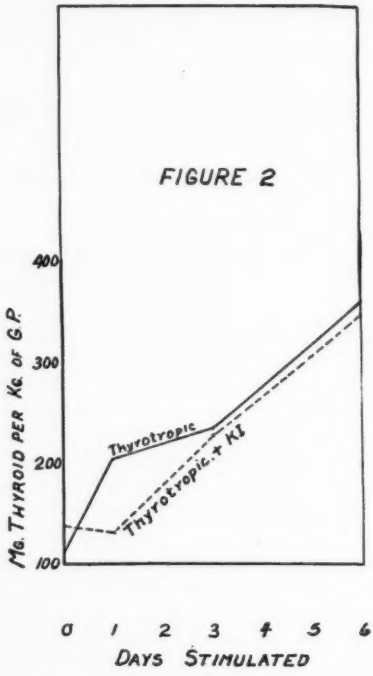
[1] Antuitrin-T, supplied by Parke, Davis & Company.
[2] To be reported later.





also administered. (The actual values of QO_2 for 11 control animals, 3 control animals given KI for 5 days, 9 animals killed after one injection of hormone, and 8 animals after one injection of hormone plus KI are given in table 1. These data show the type of variation in oxygen consumption figures encountered from animal to animal.) It will be noted from the graph that there is a marked rise in the metabolism of thyroid tissue following the injection of thyrotropic hormone, reaching a maximum of about 75% above normal within 24 hours. This increase is maintained for 3 days, following which the metabolism of the thyroid tissue decreases in spite of the continued injection of the hormone. The glands continue to hypertrophy throughout the experiment as evidenced by the progressive increase in the weight of the glands (fig. 2). Although the intraperitoneal injection of KI largely inhibits the rise in oxygen consumption of the thyroid tissue (fig. 1) caused by thyrotropic hormone, KI administration has little effect in preventing the increase in weight of the thyroid except during the first day (fig. 2).

TABLE 1. QO_2 OF THYROID TISSUE (EACH FIGURE REPRESENTS ONE ANIMAL)

	Normals		Normals KI, 5 days	Stimulated 1 day		Stimulated 1 day+KI	
	7.1	5.8	5.9	11.6	9.0	6.3	6.1
	7.0	6.4	5.2	8.8	10.1	6.4	5.7
	4.7	6.3	7.0	10.8	12.0	8.1	8.5
	5.6	6.6		8.9	7.9	10.9	5.9
	6.2	5.9		15.5			
	5.7						
Average	6.1		6.0	10.5		7.2	

The sections of thyroid tissue show evidence of marked thyroid stimulation after hormone administration. This has been partially inhibited in most animals receiving potassium iodide. Dr. Shields Warren,[3] who reviewed the sections, states that the administration of potassium iodide in these animals diminished the effect of thyrotropic hormone with respect to height of epithelium, amount of colloid absorption, number of mitoses, degree of papillary projections into the follicles, and the general histological activity of the gland. This parallels the description of human glands in spontaneous hyperthyroidism with and without iodine (16, 17).

DISCUSSION

The mode of action of iodine in toxic thyroid disease is still a somewhat debatable matter. That iodine does not inhibit the action of the circulating thyroid hormone is accepted (18). In discussing the beneficial action of iodine in toxic goiter, Eason states, "there is no proof that iodine acts as a direct sedative on the thyroid cells . . . The evidence of primary diminution of their secretory activity is lacking." Some have postulated that the primary action of iodine in ameliorating symptoms of toxic goiter is on the pituitary or midbrain. Means (17) believes that the action of iodine in toxic goiter is clearly upon the thyroid gland. We believe our results showing that iodine inhibits the metabolic response characteristic of experimentally stimulated thyroid tissue lend strong support to the contention that iodine acts directly to inhibit the metabolic activity of the thyroid gland.

SUMMARY

Thyrotropic hormone of the pituitary, administered to guinea pigs daily for 6 days, results in the following changes in the thyroid gland: (*a*) There is a marked increase in resporation of the gland which is sustained for 3 days, after which the respiration of the gland approaches normal. (*b*) There is continuous hypertrophy of the gland throughout the 6-day period.

Administration of iodine with the thyrotropic hormone largely abolishes the increased metabolism of the thyroid gland, although the gland hypertrophies to approximately the same extent as it does in the absence of iodine.

The significance of these results in relation to the therapeutic action of iodine in toxic goiter is discussed.

We wish to thank Professor A. B. Hastings for helpful suggestions in preparing this paper.

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[3] We are indebted to Dr. Shields Warren, of the Department of Pathology, of the Harvard Medical School and of the Deaconess Hospital, for histological examination of these tissues.

THYROID-OVARIAN RELATIONS[1]

I. INFLUENCE OF OVARIAN HORMONES ON THYROID HYPERPLASIA

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THE TREMENDOUS INCREASE in the use of estrogenic substances in the treatment of various functional disturbances of women has brought with it a bewildering array of claims and counterclaims regarding their efficacy. This unhappy state of affairs is due largely to our limited knowledge of the behavior of ovarian hormones in the presence of functional disturbances originating in other endocrine glands. This is particularly true as it relates to the disturbances of thyroid function, probably the commonest endocrine disturbance encountered in women during the reproductive and menopausal periods. We long have been under the impression that altered thyroid states materially affect the efficacy of a given estrogenic substance, and it seems to us that this subject deserves more intensive study along experimental as well as clinical lines. As yet, we are not in a position to present sufficient clinical data to permit conclusions. We have, however, conducted a series of experiments on white rats afflicted with a uniform type of thyroid hyperplasia and therefore well suited for the investigation of the problem under discussion.

The inception of this study dates back to an accidental observation. While surveying the thyroids of female rats used in studying possible carcinogenic effects of large doses of theelin on certain implanted tumors and on mammary and genital tissue we discovered that every thyroid was in an active state of hyperplasia. Knowing this to be contrary to the usual relation of thyroid function to prolonged or massive doses of estrogen, we looked into the various probable causes. The animals used had been implanted with mammary adenofibroma to be studied for carcinomatous changes when treated with massive doses of estrogen for long periods of time. Previous studies on the behavior of mammary adenofibromas and sarcomas in relation to endocrine function showed that the presence of these tumors did not affect thyroid behavior. Since all of our animals are housed at an automatically regulated temperature of 23.5°C. and are allowed drinking water liberally, temperature and fluid supply were eliminated as possible causes. In checking dietary data, however, we found that during seasonal depressions in breeding the technical assistant in charge of the colony had made slight changes in our stock diet by adding extra amounts of wheat germ and whole wheat in the hope of improving the fertility of the colony.[2] Since all animals fed this diet without exception showed hyperplasia of the thyroid we concluded that the change in diet had given it a goiterogenic quality. To substantiate this conclusion we surveyed the thyroids of some 200 animals of both sexes and of various ages and found

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[1] This research was supported in part by the Memorial Fund for Cancer Research and by the Rockefeller Fluid Research Fund of Stanford University.
[2] Stock diet: 10% unpurified casein, 73% corn meal, 10% linseed meal, 2% alfalfa, 3% sardine oil, 1.5% bone ash, and 0.5% sodium chloride (Walter and Addis, 1939) (1). Goiterogenic diet: 40% stock diet, 35% whole wheat, 10% wheat germ, and 15% skimmed milk.

that the degree of thyroid hyperplasia resulting from ingestion of this diet was uniform and stationary as long as this dietary regime was maintained. When it was replaced by a normal diet the thyroid hyperplasia regressed and disappeared in eight to ten days. That the thyroid modifies the effect of estrogen was called to our attention by another study conducted by us in which a number of thyrohyperplastic animals given large doses of estrogen or deprived of ovarian hormones through castration failed to show morphologic changes known to occur when the normal estrogen level is disturbed.

Discussion of the Literature

It is generally accepted that the removal of the gonads is accompanied by definite castration changes in the thyroid gland morphologically characterized by a lowering of the epithelium of the follicles and an increase in the storage of colloid. Andersen and Kennedy (2), Schultze (3), Andersen (4), Gumbrecht and Loeser (5) have described such changes in normal rats. For reasons not yet known, the thyroid responds differently when estrogen is substituted by subcutaneous administration. According to Gumbrecht and Loeser (5), subcutaneous substitution with estradiol benzoate has no effect on castration changes, while Andersen (4) found that ketohydroxyestrin restores the thyroid to normal and increases its weight. However, Gumbrecht and Loeser (5) found that when either estradiol benzoate or ketohydroxyestrin was administered by intrauterine application castration changes in the thyroid were prevented provided substitution was made at the time of castration. When castration changes were permitted to become well established before treatment, intrauterine substitution effectively restored the normal morphology of the thyroid. It therefore seems that the route used for substitution influences the ultimate effect of a given estrogen on the thyroid.

When the estrogen level of sexually intact female rats was increased above the normal for brief periods not exceeding five or six days, a transitory increase in thyroid activity was observed (6, 7, 8). When, however, estrogen was administered for long periods the thyroid presented definite evidence of atrophy (5–12). This seems to indicate that the length of the period of treatment also is an important factor in the ultimate effect upon the thyroid.

Relatively little is known of the relation of the corpus luteum to the thyroid. Knaus (13) reported in 1923 that progesterone was capable of depressing the function of the thyroid of normal rats as indicated by the storage of colloid. In 1936 and 1938 respectively De Amilibia et al. (8) and Gumbrecht and Loeser (5) described experiments which failed to substantiate Knaus' findings. Comparatively recently (1939) Alexiu (14) presented evidence that progesterone stimulates the thyroids of normal as well as castrated female rats. These contradictory findings can only mean that the effect of progesterone on the thyroid, like that of estrogen, is subject to modifications which require further clarification.

All findings discussed in the previous paragraphs deal with animals with normal thyroids. In the following paragraphs we shall discuss observations dealing with the abnormal thyroid and its relation to ovarian hormones.

In 1928 Reiss and Pereny (15) demonstrated that the feeding of "thyroid hormone" to castrates inhibited parenterally administered estrogen. A few years later van Horn (16) reported that it required three times more theelin to eliminate vaginal castration changes in mature female rats in a state of hyperthyroidism produced by desiccated thyroid than it did in castrates with relatively normal thyroids. He concluded that the increased metabolism incident to the feeding of thyroid substance increased the speed of estrogen elimination. Weichert and Boyd (17) in 1933 found that diestrus was prolonged when rats were fed desiccated thyroid from 42 to 53 days. This they found to be accompanied by the formation of unusually large corpora lutea such as are seen in pregnancy. They demonstrated the functional activity of these bodies by showing that irritation of the endometrium by the insertion of threads led to the formation of what they called "placentomata." The explanations given were that either the excessive supply of thyroid hormone stimulated the anterior pituitary to the production of unusual amounts of luteinizing hormone, or that the increased metabolism depressed the estrin level, permitting the persistence and over-development of corpora lutea. In line with this explanation, Meyer and Wertz (18) stated that the demand for

estrin rises with the increased supply of thyroid hormone. All of the last evidence cited points to the importance of the degree of thyroid function in its relation to estrogen utilization. That thyroid hormone can alter the action of pituitary gonadotropins was demonstrated in our laboratory by Fluhmann in 1934 (19). He found that hyperthyroidism artificially induced by thyroxin or desiccated thyroid inhibited the action of the follicle stimulating hormone, while hypothyroidism resulting from thyroidectomy increases the effect of parenterally administered pituitary gonadotropic hormones on the ovaries and the uterus.

In the foregoing paragraphs we have discussed pertinent factors dealing with the effect of withdrawal and substitution of estrogen and progestogen on the normal thyroid. We also have described changes in this relationship resulting from hyperthyroidism induced by feeding thyroid extract or substances, or by the administration of thyroxin. In the following experiments we shall discuss the thyroid-ovarian relationship as influenced by thyroid hyperplasia induced by a goiterogenic diet. Although we have made no iodine determinations we assume that the type of hyperplasia we are dealing with in these experiments is the same as described for simple goiter and signifies an attempt on the part of the thyroid to compensate for a relative or absolute iodine deficiency. In this state, thyroid hyperplasia and hypertrophy are a part of a defense mechanism against the effect of a faulty diet and therefore diametrically different from thyroid conditions resulting from excessive thyroid hormone production or supply. In a series of experiments we studied various phases of the relationship between thyroid hyperplasia and ovarian hormones. Our first report deals with the withdrawal of ovarian hormones and substitutional therapy with ketohydroxyestrin and progesterone.[3]

Experiment I

Seventy female white rats of our own strain were castrated at the age of 30 days. All were offspring of mothers fed on the goiterogenic diet and were put on the diet after weaning for the duration of the experiment. The animals were divided into three groups, 20 non-castrated animals serving as controls, 20 of the castrates were not treated, and the remaining 30 castrates were given ketohydroxyestrin and progesterone as outlined below. Eleven animals died during the course of the experiment. In the treated group, death usually was due to abscess formation at the site of injection.

At the age of 170 days, 10 rats of the third group were given 200 I.U. of ketohydroxyestrin in oil 5 times a week until a total of 10,000 U had been given. Ten others received 0.2 I.U. of progesterone 5 times a week until 10 U had been given, and 10 were given both substances in the amounts stated above. Injections were made subcutaneously and on the whole were tolerated reasonably well.

We made the following observations on the morphologic changes of the thyroid gland in the 3 groups. The thyroids of all control animals presented a fairly uniform macroscopic enlargement. Microscopically, the glands had changed into a state of hyperplasia and hypertrophy, affecting both the parenchyma and the stroma (fig. 1). Comparative studies left us with the impression that hyperplasia was uniform and that no spontaneous regression occurred as long as the animals were fed on this particular diet. Withdrawal of ovarian hormones by castration failed to affect the state of hyperplasia to any appreciable degree (fig. 2). When estrogen was substituted in the form of Theelin in oil in the dosages stated, all castration changes of the genital tract, breasts and hypophysis disappeared. However, thyroid hyperplasia was in no way affected (fig. 3). On the other hand, progesterone given in the form of Lipo-Lutin did not eliminate castration changes but significantly reduced the state of hyperplasia

[3] The ketohydroxyestrin, in the form of Theelin, and the progesterone, in the form of Lipo-Lutin, were supplied by Parke, Davis & Company, Detroit, Michigan, through the courtesy of Dr. E. A. Sharp.

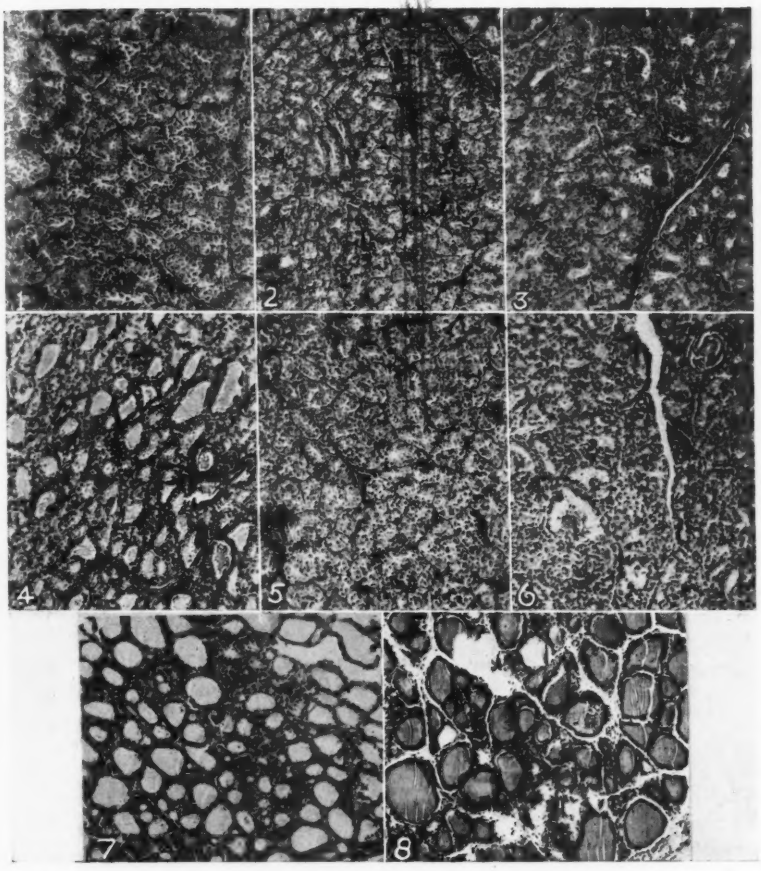

Fig. 1. Diffuse hyperplasia and hypertrophy of thyroid gland of female rat induced by diet. (×85) Fig. 2. Effect of castration on hyperplastic thyroid. (×85) Fig. 3. Effect of castration and estrogenic replacement therapy on hyperplastic thyroid. (×85) Fig. 4. Effect of castration and replacement therapy with estrogen and progestogen on hyperplastic thyroid. Note lowered epithelium and evidence of colloid storage. (×85) Fig. 5. Effect of small doses of estrogen on hyperplastic thyroid of sexually intact female rat (1,200 I.U.). (×85) Fig. 6. Effect of massive doses of estrogen on hyperplastic thyroid of sexually intact female rat (86,000 I.U.). (×85) Fig. 7. Thyroid of female rat control. (×85) Fig. 8. Effect of castration on thyroid of female control. (×85).

of the thyroid gland. The follicular epithelium was not only lowered, but storage of colloid in significant amounts occurred. When Theelin and Lipo-Lutin were administered in combination, hyperplasia of the thyroid was reduced in the same degree as when Lipo-Lutin was given alone (fig. 4). In addition, castration changes disappeared as readily as when estrogen was given alone.

Results. No castration changes occurred in the hyperplastic thyroid. Substitution by ketohydroxyestrin in amounts sufficient to eliminate castration changes in the genital organs, breasts, and pituitary gland had no effect on the hyperplastic state of the thyroid gland of castrated female rats of our strain. Progesterone inhibited thyroid hyperplasia in such rats but failed to affect castration changes. The addition of keto-

hydroxyestrin to progesterone did not alter the effect of the latter on the thyroid nor of the former on the genital tract and breasts.

Experiment II

One hundred and one female rats 130, 240, and 400 days old, borne by mothers fed on a goiterogenic diet and themselves put on this diet after weaning, were studied for the effect of varying dosages of estrogen on thyroid hyperplasia in the presence of normally functioning ovaries. Twenty-seven animals were set aside as controls, 44 were given 100 I.U. of ketohydroxyestrin per week, and the other 30 were given 1,000 I.U. per day. The group of 44 were all 130 days old at the beginning of treatment. Seventeen of them were given the dosage in bi-weekly injections and 27 in weekly injections. The ultimate dosage in 17 rats totalled 1,200 to 1,400 U; in 11, 1,400 to 1,600 U; and in 16, 1,600 to 1,900 U.

The 30 rats receiving 1,000 I.U. daily were divided into 3 groups. With 10, treatment was begun at the age of 130 days, with 5 at 140 days, and with 15 at 400 days. The period of treatment varied from 14 to 127 days, the animals being killed at intervals in order to observe the effect of various dosage levels.

Results. Ketohydroxyestrin in dosages of 100 I.U. either by bi-weekly or mono-weekly injection over a period of from 12 to 19 weeks did not affect thyroid hyperplasia induced by a goiterogenic diet in mature sexually-intact female rats of our colony (fig. 5). When, however, it was given in large doses of 6,000 U per week more than half the animals developed focal necrosis in the hyperplastic thyroid. This usually occurred after a total of 50,000 to 60,000 I.U. had been given. When necrosis was permitted to progress under additional loads of ketohydroxyestrin, the weight of the thyroid decreased proportionately to the increase in the dosage. Morphologically this was characterized by progressive hyalinization and fibrosis of the thyroid and in many ways corresponded to the exhaustive atrophy of the thyroid resulting from sustained hyperactivity without periods of physical rest (fig. 6) (11). These changes were not uniform in extent or severity and in three instances the hyperplasia remained unaffected. These animals received 100,000 to 110,000 U of ketohydroxyestrin over a period of 115 to 127 days and their thyroid hyperplasia differed in no way from that of untreated animals. Certain observations concerning the morphology of the pituitary gland, to be published subsequently, leave us with the impression that the hypophysis harbors the important factor governing the effect of large doses of ketohydroxyestrin on the thyroid hyperplastic gland. We are certain that the variations in the reaction of the thyroid hyperplasia to massive doses of estrogen cannot be explained by the age factor.

DISCUSSION

Previous to the experiments reported here we studied a large group of female rats of all ages with hyperplasia of the thyroid gland resulting from a goiterogenic diet. The degree of hyperplasia resembled simple goiter and was uniformly maintained as long as the diet was continued. Neither exhaustion atrophy nor colloid nor resting stages was encountered. In this respect the thyroid hyperplasia produced by us differed from the known behavior of simple goiter. The reason for this may be 2-fold. Either the underlying deficiency factor is too mild to create an exhaustion atrophy or its continued presence prevents a return to a resting or a colloid stage. The normal thyroids of the females of our colony respond to castration by morphologic changes indicative of lowered thyroid function (fig. 7, 8). These changes are readily eliminated by estrogenic substitution therapy. After hyperplasia occurs, however, the thyroids no longer show morphologic castration changes although the genital tract, breasts

and hypophysis react in the usual way. Substitutional therapy with ketohydroxyestrin eliminates all castration changes of the latter organs but has no effect upon thyroid hyperplasia. When castrates with thyroid hyperplasia were given progesterone parenterally the hyperplasia was significantly reduced. Castration changes, however, as described above, were not affected. The addition of ketohydroxyestrin to progesterone did not increase the action of the corpus luteum hormone on the thyroid but corrected the genital castration changes. It therefore is of interest that substitutional therapy with comparatively small doses of progesterone beneficially affects the degree of thyroid hyperplasia. It also is of interest that thyroid hyperplasia of either castrated or sexually intact females is not influenced by moderate amounts of ketohydroxyestrin. However, the continuous administration of large amounts over a period of time can damage the thyroid gland, as manifested by various morphologic changes. We doubt that this is a characteristic of ketohydroxyestrin. It probably will be found to be true of any substance that can affect the action of the anterior pituitary. It has been demonstrated that large amounts of estrogen inhibit the effect of thyrotropic hormones when the two hormones are given simultaneously (20). It also has been shown that when animals are pretreated with thyrotropic hormones subsequent administration of an estrogen given in large doses over comparatively short periods is incapable of decreasing established thyroid hyperplasia (5, 10). This is in keeping with our own observations. The animals studied by us had been subjected to thyroid hyperplasia for many weeks and therefore corresponded in many respects to animals pretreated with thyrotropic hormones. Like the latter, large doses of an estrogen given over short periods had no effect upon the hyperplasia. It is therefore important that even well established hyperplasia can ultimately be disturbed if estrogen therapy is carried out long enough with sufficiently large doses. The resulting morphologic picture is that of an exhaustion atrophy. The most probable explanation is that the long-continued use of an estrogen damages the function of the anterior pituitary gland, in which case the thyroid as well as the ovaries will suffer. We will show in a subsequent report that this is correct.

The fact that thyroid hyperplasia in castrates is reduced after administration of progesterone is exceedingly interesting. We have stated that the effect is uniform, but as yet we are ignorant of the mechanism involved. The studies related here uniformly indicate that the utilization of estrogen is largely influenced by the functional state of the thyroid, and it is reasonable to assume that this is not the only endocrine gland which has this characteristic. It is not at all unlikely that the endocrine apparatus in man is subject to similar relations. The need for clinical observations along these lines is evident.

SUMMARY

Thyroid hyperplasia is readily induced in rats, regardless of age, by a goiterogenic diet.

Withdrawal of ovarian hormones by castration of rats with normal thyroids produces morphologic evidence of decreased thyroid activity.

Withdrawal of ovarian hormones by castration of rats with hyperplastic thyroids does not affect the degree of hyperplasia.

Thyroid hyperplasia in castrates is not affected by parenteral substitution of estrogens.

Thyroid hyperplasia in castrates is reduced by parenteral substitution of progestogen alone or in combination with an estrogen.

Thyroid hyperplasia in sexually intact female rats is not affected by moderate amounts of an estrogen.

Thyroid hyperplasia in sexually intact animals is accompanied by an exhaustion atrophy when large doses of an estrogen are administered.

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THYROID-OVARIAN RELATIONS[1]

II. THE EFFECT OF CASTRATION AND REPLACEMENT THERAPY ON THYROID, PITUITARY, ADRENAL AND BODY WEIGHT IN THYROHYPERPLASTIC ALBINO RATS

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THE MORPHOLOGICAL BEHAVIOR of thyroid glands of rats fed a goiter-producing diet in its relation to the action of ovarian hormones was described in a previous report (1). It was shown that for the duration of feeding neither withdrawal of ovarian hormones nor replacement with ketohydroxyestrin (subsequently referred to here as estrone) influenced thyroid hyperplasia in castrated rats. The administration of progesterone to such rats, however, decreased thyroid hyperplasia materially.

Since in that paper the considerations were purely morphological, the present report furnishes additional information on weight changes in the thyroid, pituitary and adrenal glands, and in the body as a whole.

PROCEDURE

Seventy 30-day-old thyrohyperplastic female rats of the Wistar strain were

TABLE 1. GROUPS AND TREATMENTS

Group	Symbol	Original No.	Died	No.	Treatment (over a period of 10 weeks)
I	C	20	2	18	Control
II	Ca	20	2	18	Castrated
III	Ca E	10	3	7	Castrated. Received 200 I.U. estrone[1] in oil 5× weekly or a total dose of 10,000 I.U.
IV	Ca P	10	1	9	Castrated. Received 0.2 I.U. progesterone[1] in oil 5× weekly or a total dose of 10 I.U.
V	Ca EP	10	3	7	Castrated. Received combined dosages of above, i.e., a total of 10,000 I.U. estrone plus 10 I.U. progesterone
		70	11	59	

[1] The estrone and progesterone used were Theelin and Lipo-lutin respectively, furnished through the courtesy of Parke, Davis & Company, Detroit, Mich.

divided into 5 groups as shown in table 1. The animals included in 4 of the groups were castrated at this time, the remaining group being reserved for control. At 140

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days after castration (i.e., age = 170 days), treatment was instituted according to the schedule given in the table, and was continued thereafter for a period of 70 days, when the animals were killed and the various weight determinations made.

During this time 11 animals died, necessitating the corrections for numbers given in the table.

Analysis

The usual statistical constants were calculated for the gland weights as shown in table 2, and for the body weight in table 4. It should be noted that body weight

TABLE 2. CALCULATED CONSTANTS (Weights given in mg.)

Treatment		C[1]	Ca	Ca E	Ca P	Ca EP
No. of Animals		18	18[2]	7	9	7
Thyroid	Mean	40.61	36.39	37.29	28.22	27.57
	St. Dev.	11.10	10.71	12.96	5.76	9.86
	St. Error	2.61	2.52	4.90	1.92	3.73
Pituitary	Mean	13.94	14.94	18.00	15.11	15.29
	St. Dev.	1.95	1.85	3.83	1.46	2.55
	St. Error	0.45	0.45	1.45	0.49	0.96
Adrenal	Mean	52.33	45.33	61.86	43.56	52.43
	St. Dev.	6.34	8.21	11.39	11.69	10.94
	St. Error	1.50	1.94	4.31	3.90	4.13

[1] See table 1, col. 2.
[2] Pituitary—Ca Group: N = 17.

changes were calculated on the basis of percentage increase or decrease of autopsy weight over weight at the beginning of the experiment (i.e., 140 days after castration),

TABLE 3. SIGNIFICANCE OF DIFFERENCES (D)[1] BETWEEN MEANS (Differences given in mg.)

Means compared		Thyroid			Pituitary			Adrenal		
$\bar{x}_1$	$\bar{x}_2$	D	t[2]	P[3]	D	t	P	D	t	P
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
C	Ca	−4.22	1.161	.20	+1.00	1.559	.10	−7.00	2.863	<.01
	Ca E	−3.32	0.643	.50	+4.06	3.554	<.01	+9.53	2.681	<.02
	Ca P	−12.39	3.124	<.01	+1.17	1.598	.10	−8.77	2.549	<.02
	Ca EP	−13.04	2.713	<.02	+1.35	1.431	.10	+0.10	0.028	.90
Ca	Ca E	+0.90	0.176	.80	+3.06	2.743	<.02	+16.53	4.055	<.001
	Ca P	−8.17	2.125	.045	+0.17	0.238	.80	+1.77	0.460	.60
	Ca EP	−8.82	1.886	>.05	+0.35	0.372	.70	+7.10	1.770	>.05
Ca E	Ca P	−9.07	1.886	>.05	−2.89	2.039	>.05	−18.30	3.139	<.01
	Ca EP	−9.72	1.578	.10	−2.71	1.561	.10	−9.43	1.579	.10
Ca P	Ca EP	−0.65	0.166	.80	+0.18	0.174	.80	+8.87	1.548	.10

[1] $D = x_2 - x_1$.
[2] t = difference divided by its standard error.
[3] P = probability of occurrence by chance alone.

because, as is well known, the variation between individual rat weights, however great the degree of control, is always large.

Evaluation of the effects of treatment was made on the basis of Fisher's (2) method for determining the significance of differences between means.

In each case a "null" hypothesis is set up to the effect that: (*a*) there is no difference between the means of the various groups compared, or, in the case of body weights, (*b*) the means of the percentage changes do not differ from zero.

The results of the calculations are expressed in probabilities, the usual standard

TABLE 4. CHANGES IN BODY WEIGHT (Means given in %)

Constants	C[1]	Ca	Ca E	Ca P	Ca EP
Mean	+0.04	+6.43	−10.67	+6.05	−16.11
St. Dev.	4.93	5.21	6.36	14.14	10.75
St. Error	1.16	1.23	2.40	4.80	4.06
t[2]	0.034	5.234	4.442	1.260	3.964
P[3]	.90	<.001	<.01	.20	<.01

[1] See table 1, col. 2.
[2] t = mean difference divided by its standard error.
[3] P = the probability that the means differ significantly from zero.

of significance being P ≷ .05. In other words, a P value of, say, .02 indicates that in only two cases out of a hundred would the observed difference have occurred as a result of chance alone, hence the 'null' hypothesis is regarded as not proven and the difference considered significant. In fact, a P value of .02 or less is considered highly significant.

As the method takes into account the possible small size of the sample, as well as the relatively large standard deviations often associated therewith, reliance may be placed on the conclusions drawn.

The results are shown in tables 3 and 4; and it should be noted that the calculations in these tables as well as those in table 2 were carried out to a greater number of decimal places than are given here.

Effects of Treatment

On the thyroid. Neither castration nor subsequent replacement with estrone affected thyroid weights. Progesterone, however, when injected into castrated animals, both alone and simultaneously with estrone resulted in decreases in weight of a like order. Thus the effect was due to progesterone and not estrone, which is confirmed by the fact that the thyroids of castrated animals treated with progesterone alone showed a significant decrease in weight when compared to untreated castrates.

On the pituitary. No effect was noted as a result of castration alone, but replacement of estrone in castrates increased pituitary weights. Neither did injection of progesterone alone affect the weight, but when given in combination with estrone the replacement effect of the latter was apparently inhibited.

On the adrenal. Castration alone, as well as the administration of progesterone to castrates, resulted in a decrease in weight of the same order, from which it follows that the effect was caused by castration and not progesterone.

Estrone, on the other hand, when injected into castrates, caused an increase in weight even above that of the non-castrated controls, which effect was apparently nullified by administration of the two hormones simultaneously. Thus, in our animals, the estrone effect was inhibited by progesterone.

On body weight. The constants are given in table 4.

During the period of the experiment the body weight of the control animals did not change. This was to be expected, normal increment in weight being inhibited by the thyrohyperplastic state of the animals.

In spite of this condition, however, castration resulted in obesity, fat deposits being found particularly in the subcutaneous and retroperitoneal tissues. The mean increase in weight of these animals was 6.4%.

The injection of estrone reversed the trend, the corresponding mean weight loss being 10.7%, while the addition of progesterone at the same time gave an apparent still further decrease, which, however, was not significant, the difference between these two means being -5.437%, $t = 1.152$ with P being greater than .20. Taken by itself, however, the administration of estrone and progesterone simultaneously caused a decrease of 16.1% in body weight.

The change resulting from the injection of progesterone into castrates was of no significance.

It should be noted that the weight losses observed were due, in part at least, to the frequency of the injections as well as to the toxic effects of injection abscesses. But even taking these factors into account, it is probable that the estrone effect is a true one.

DISCUSSION

Body weight and thyroid weight. It has been demonstrated that, while castration obesity in rats with thyroid hyperplasia may be over-corrected into an actual weight loss through the administration of estrone in the doses used, there is no change in weight of the thyroid itself during either the period of body weight increase or decrease. On the other hand, progesterone, which decreases thyroid weight, has no significant effect on the obesity. Obesity thus appears a function of estrogen withdrawal rather than one of thyroid activity (as measured by weight) regardless of any changes which might take place in the latter.

This is in complete agreement with our previous observations on the morphology of hyperplastic thyroids, where the maintenance of the hyperplastic state following castration was reported. The importance of the estrogenic factor in relation to the development of castration obesity remains unchanged, even in rats with thyroids hyperplastic to begin with.

Adrenal weight. When performed at an immature age, castration decreases adrenal weights in thyrohyperplastic rats. This agrees with the observations of Hatai (3) and Blumenfeld (4), while Andersen and Kennedy (5) did not observe significant changes in the adrenal weight 9 weeks after the castration of normal rats. That the decrease found in our animals was due to lack of estrogen is demonstrated by the increase of adrenal weight following the administration of estrone.

On the other hand, progesterone, when given simultaneously with estrone, counteracted the effect of the latter. This inhibitory effect of progesterone on estrone, when applied to the adrenals of castrated females, is paralleled by the observations of Selye (6) on non-castrated male rats, in which progesterone decreased the effect of estradiol on the adrenals.

The adrenal response of castrated female rats to estrogen frequently has been studied with varying results. Enlargement has been reported (7–10) in mature castrated females. No effects were noted in immature animals (11) in mature castrates (12, 13). These inconsistent results are apparently due to variations of procedure, especially with regard to postoperative periods and estrogen doses employed.

Pituitary weight. The increase occurring in our immaturely castrated thyrohyperplastic females following estrogenic treatment is of interest, as it has not been reported following the treatment of immature non-castrated females over short periods (14, 15, 16). Neither did Zondek (17) find an increase in pituitary weights following estrogen administration for periods of less than 4 months, but when the treatment

was continued further, an increase was noted. Progesterone counteracted the effect of estrone on pituitary weight (as was observed in our adrenals) so that no significant difference was found following simultaneous injection of these two hormones.

SUMMARY

In thyrohyperplastic female rats: (*a*) castration had no effect on thyroid or pituitary weights, decreased adrenal weight, and increased body weight; (*b*) estrone increased pituitary and adrenal weight, had no effect on thyroid weight, and more than compensated for the increase of body weight due to castration; (*c*) progesterone decreased thyroid weight, but had no effect on the pituitary, adrenal or body weights; (*d*) estrone and progesterone given simultaneously had an effect on the adrenal and pituitary only in that the action of the two hormones appeared to nullify one another.

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THYROID-OVARIAN RELATIONS[1]

III. MORPHOLOGIC OVARIAN CHANGES IN ESTROGEN TREATED THYROHYPERPLASTIC RATS

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IN A PREVIOUS PAPER (1) we reported that the responses of hyperplastic thyroids of rats to the withdrawal, substitution and superimposition of estrogen and progestrogen differ from those of normal thyroids. We are reporting here observations indicating that the effect of an estrogen on the ovaries of rats afflicted with thyroid hyperplasia differs from that on the ovaries of rats with normal thyroids.

It is well known that the cessation of anterior pituitary function is followed by abrupt ovarian changes. Morphologically this is characterized by three distinct phases: failure of primordial follicles to mature, persistence of corpora lutea (2), and typical and constant changes in the appearance of the cells of the interstitial apparatus (3).

Only replacement therapy by means of gonadotropic substances can arrest or reverse these changes (2, 3). It may be taken for granted, therefore, that the presence of morphologic changes in the ovaries similar to those found after hypophysectomy are indicative of pituitary deficiency. We recently found such changes in the ovaries of nonhypophysectomized rats afflicted with thyroid hyperplasia and treated parenterally with estrogen. From this we deduced that thyroid hyperplasia had disturbed the normal pituitary-thyroid-ovarian relations.

To obtain further information we studied the effect of thyroid hyperplasia on the ovaries with and without additional estrogen supply. Twenty-seven white female rats were put on a goiterogenic diet when weaned and allowed to reach an age of 189 to 301 days. At autopsy they all showed uniform and well developed thyroid hyperplasia (fig. 1). However, the ovaries revealed no morphologic changes, although irregularities in the estrous cycle occurred (fig. 2). To determine whether or not age was a factor, a group of 9 females was allowed to reach an age of 332 to 471 days. In other words, they were kept on this goiterogenic diet for a period of 8 months or longer. The thyroids removed from these older rats also showed uniform hyperplasia and morphologically normal ovaries for their ages. From this we concluded that moderate thyroid hyperplasia induced by a goiterogenic diet, regardless of its duration and the age of the host, does not produce a deficiency reaction in the ovaries. From this it can be deduced that in rats the anterior pituitary apparatus is ordinarily not disturbed by moderate thyroid hyperplasia.

As far as the ovaries of rats with normal thyroids are concerned, it is known that they respond differently to various dosages of estrogen. Large doses, particularly

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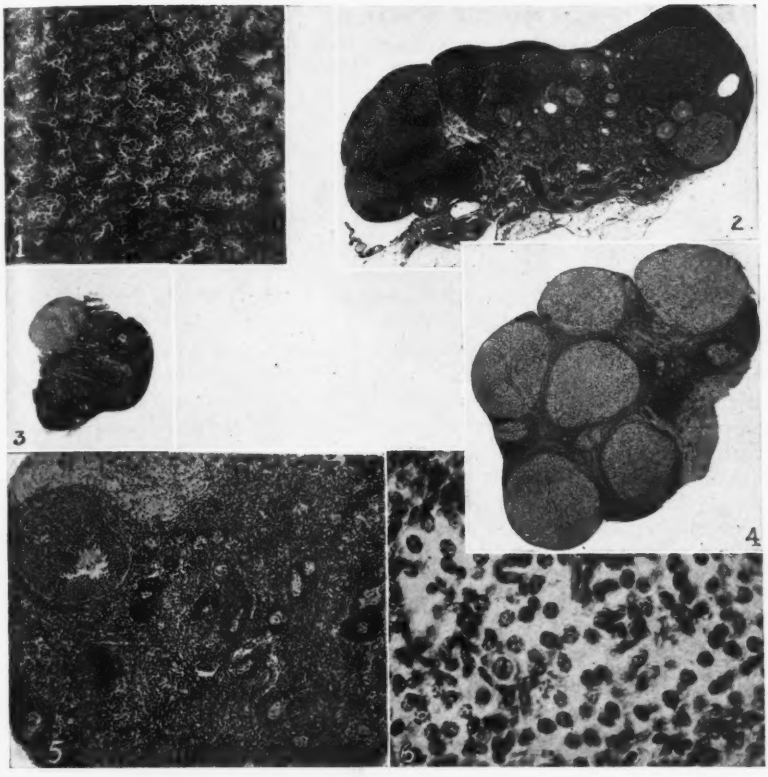

Fig. 1. THYROID HYPERPLASIA AND HYPERTROPHY induced by diet in a 194-day-old female rat (×78). Fig. 2. OVARY OF THYROHYPERPLASTIC RAT 189 days old (×9). Fig. 3. OVARIAN ATROPHY in 240-day-old thyrohyperplastic rat after 86,000 I.U. of theelin over 100 days (×9). Fig. 4. OVARY of 493-day-old thyrohyperplastic rat after 1,500 I.U. of theelin over 99 days. Persistent corpora lutea; inhibition of follicular growth. Interstitial tissue shows deficiency reaction (×9). Fig. 5. OVARY of 481-day-old thyrohyperplastic rat after 1,000 I.U. of theelin over 66 days. Note follicular atresia and increased interstitial tissue (×78). Fig. 6. HIGH POWER OF FIG. 5. Deficiency reaction of interstitial tissue (×313).

when given daily, produce a rapid atrophy in our rats (fig. 3), while small doses fail to do so.

In the following experiments it will be shown that under certain circumstances prolonged hyperplasia of the thyroid can modify the effect of parenterally supplied estrogen on the ovaries.[2]

Twelve 400-day-old female rats afflicted with thyroid hyperplasia were injected once a week with 100 I.U. of theelin in oil for a period of 7 to 17 weeks. The first animal was killed after a total of 800 I.U. had been administered. Nine were subsequently killed at intervals, and 2 were allowed to recover for 40 and 47 days respectively after discontinuation of treatment. We found the following interesting changes. Eight of the animals killed at weekly intervals after having received a total of 1,000 to 1,500 I.U. of theelin presented ovaries which were characterized by follicular atresia with

[2] The ketohydroxyestrin, in the form of Theelin, used in these experiments was supplied by Parke, Davis & Company, Detroit, Michigan, through the courtesy of Dr. E. A. Sharp.

abortive development of some of the follicles at the antrum stage. Persistent corpora lutea were present in various stages of arrest (fig. 4). In 2 animals, ovarian atrophy had advanced to such a degree that all evidence of luteinization had disappeared. It seemed to us that the interstitial cells had increased in number, but we are not certain whether this increase was actual or relative (fig. 5). There was, however, the typical transformation of interstitial cells into wheel-like cells, described as indicative of pituitary failure. These cells had assumed all of the characteristics described for such cells; that is, poor staining quality of the cell protoplasm, shrinkage of the cell mass, and arrangement of the nuclear chromatin into a wheel-like structure (fig. 6). Most of these ovaries showed a complete transformation of the interstitial apparatus, although in some instances it was more marked in the vicinity of the atretic follicles. In general, the changes observed corresponded in every way to those seen in the ovaries after destruction of the pituitary gland.

Twenty-six young female rats averaging 120 days of age, with thyroid hyperplasia, were given 100 I.U. of theelin in oil once a week for a period of 7 to 17 weeks. Twenty-five of these animals presented perfectly normal appearing ovaries, while only one, having received 1,400 I.U. over a period of 13 weeks, showed changes in the interstitial apparatus as described for older rats. It is evident, therefore, that in the presence of thyroid hyperplasia the ovaries of young rats respond differently to an estrogen than do the ovaries of very mature animals. Taking for granted that follicular atresia, the persistence of corpora lutea, and the transformation of interstitial cells into the so-called wheel or deficiency cells indicate pituitary failure of some kind, the above findings suggest that in older rats comparatively small doses of an estrogen can depress pituitary function when thyroid hyperplasia has been well established.

SUMMARY

It appears to us that age is an important factor in relation to estrogen therapy in the presence of altered thyroid behavior, possibly because an estrogenic overload depressing the gonadotropic apparatus occurs more rapidly in the presence of thyroid hyperplasia. The latter explanation fits in with Fluhmann's observations (4), which revealed that the action of pituitary gonadotropin can be depressed when thyroid activity is increased by the administration of thyroxin or desiccated thyroid. The interesting phenomenon observed in older rats is not of a permanent nature, since the ovaries recover after the discontinuation of estrogen therapy. In calling attention to the importance of thyroid hyperplasia in relation to estrogen treatment, we have in mind that such an observation may be of clinical value, particularly in that period of a woman's life which concerns itself with the climacterium when thyroid disturbances are common and estrogen therapy finds its greatest application.

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REFRACTORINESS PRODUCED BY SODIUM RETAINING SUBSTANCES[1]

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IN PREVIOUS WORK from this laboratory it was shown that repeated intravenous injections of adrenal extract lead to refractoriness to sodium retaining effects of the extract (1). The refractory state is species specific (2). After refractoriness has been developed in an adrenalectomized animal the amount of extract required for maintenance is increased. During refractoriness the response to desoxycorticosterone is undiminished. Moreover it is impossible to develop refractoriness from injections of corticosterone (3).

In the present work we have continued the study of sodium retaining substances and their ability to confer refractoriness.

METHODS

The subjects were normal adult female dogs. The care of the animals and the analytical methods have been described elsewhere (2).

Materials for injection were prepared as indicated. Corticosterone was dissolved in 10% ethyl alcohol. Desoxycorticosterone was dissolved in 5% ethyl alcohol.

Sodium factor precipitate was prepared by the petroleum ether-ethylene dichloride method (4). The precipitate was dissolved in 10% ethyl alcohol.

Beef adrenal gland protein was prepared from a standard extract of whole glands in 10% ethyl alcohol. The sodium factor was removed from the extract by ethylene dichloride. After removal of the alcohol, insoluble material was discarded. The aqueous solution contained the protein. There was no detectible amount of sodium factor remaining. This was determined by injecting 5 times the usual amount of material.

Two cc. of a 0.037% solution of desoxycorticosterone in 5% alcohol was mixed with 0.4 cc. of pig serum being kept at 35°C. for one hour before injection.

Two cc. of a 0.03% solution of desoxycorticosterone in 5% alcohol was mixed with 0.4 cc. of adrenal protein solution being kept at 35°C. for one hour before injection.

Four cc. of a 0.15% solution of corticosterone in 10% ethyl alcohol was mixed with 0.4 cc. of adrenal protein solution being kept at 35°C. for 1 hour before injection.

Injections were made intravenously at the beginning of the 6-hour test period.

RESULTS

Effect of precipitate or crystalline preparations. When an attempt is made to separate sodium factor by precipitation from other active substances in adrenal extracts a definite change takes place. The precipitate loses the power to confer refractoriness

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although its ability to cause sodium retention appears undiminished. One experiment illustrates this very clearly. A dog responded to a certain dose of sodium factor precipitate by 30% retention of sodium on the first injection. Eight intravenous injections of the same dose failed to cause any refractoriness, the sodium retention from the last injection being 28%. When whole adrenal extract is used, 4 or 5 intravenous injections are adequate to produce complete refractoriness (2).

Corticosterone and desoxycorticosterone act in a manner similar to precipitated sodium factor. Repeated injections fail to produce the refractory state. The initial injection of 4 mg. of corticosterone caused a 29% retention of sodium. A fourth injection of the same dose caused 27% retention. The first injection of 0.6 mg. of desoxy-

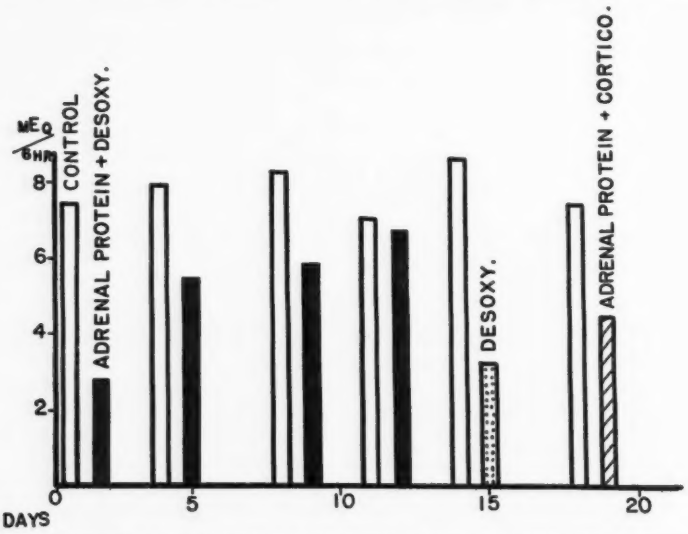

Fig. 1. Excretion of sodium during a 6-hour period in response to intravenous injections of sodium retaining substances. Injections of 0.6 mg. desoxycorticosterone plus adrenal protein, in solid black. Injection of 0.6 mg. desoxycorticosterone, dotted. Injection of 6 mg. corticosterone plus adrenal protein, diagonal lining.

corticosterone caused a 61% retention of sodium. A fifth injection of the same dose gave a 64% retention.

Effect of protein combinations. Repeated intravenous injections of desoxycorticosterone plus pig serum led to the development of partial refractoriness. The first injection caused 52% retention of sodium. The fourth injection caused 32% retention while the sixteenth injection caused 29% retention. The pig serum-desoxycorticosterone mixture was incubated at 35° for one hour, before injection in the first 11 experiments. In order to insure sufficient time for interaction the incubation period was extended to 18 hours for the last 5 injections. The injection of desoxycorticosterone alone into the partially refractory animal elicited a normal response (50% retention of sodium).

Repeated intravenous injections of the desoxycorticosterone beef adrenal protein mixture led to the development of complete refractoriness. The results in one dog are shown in figure 1. There was 62% retention of sodium from the first injection and no significant retention from the fourth injection. However, desoxycorticosterone

alone gave 63% retention. Moreover the animal was responsive to a corticosterone-adrenal protein mixture.

A second animal gave similar results. In this dog after refractoriness to beef adrenal protein-desoxycorticosterone mixture had developed, the effect of pig serum-desoxycorticosterone mixture was tried. The response was just as great as that for desoxycorticosterone alone. Therefore the refractoriness is extremely specific.

The repeated injection of adrenal protein solution alone failed to cause the development of refractoriness to the adrenal protein-desoxycorticosterone mixture.

Precipitin reaction. After the development of refractoriness blood was drawn from the dogs and the serum separated. Precipitin tests were made using the ring method. The desoxycorticosterone-pig serum and desoxycorticosterone-beef adrenal protein combinations were used as antigens. An attempt was made to demonstrate a blocking reaction by overlaying the serum with desoxycorticosterone solution before the addition of the "combination." Sera from untreated normal dogs were used as controls.

The sera of the 2 dogs tested gave a positive precipitin test when the desoxycorticosterone-protein mixture to which they were refractory was used as antigen. The titer was low, being positive only in dilution 1:10. No blocking reaction could be demonstrated. No reaction was visible when desoxycorticosterone solution was used alone.

DISCUSSION

Despite the limited number of experiments reported, the numerous tests in each experiment and the uniformity of the responses make a greater number unnecessary.

Our results show that the known crystalline sodium retaining substances or the precipitated sodium factor will not in themselves develop refractoriness. They can do so if combined with a protein.

Taking the desoxycorticosterone-protein combinations as an illustration, it is notable that an adrenal protein combination is more effective than a serum protein combination, the former developing complete refractoriness.

The method employed for the preparation of adrenal protein did not preclude the presence of cortin, therefore, it is remotely possible that cortin plays some part in the development of the refractory state.

There are indications that the sodium factor is not desoxycorticosterone. The latter is more stable and more easily crystallized. Whether the two substances are identical or not, the sodium retaining power in the adrenal appears to reside in a compound or compounds which is, or are, able to confer refractoriness when associated with protein material. Proof of this is found in the observation that in adrenalectomized animals the use of an adrenal extract to which it has been made refractory, has no influence on sodium retention. That is, the sodium concentration in the plasma is low, 130 m.Eq. per liter and is typical of that observed in adrenal insufficiency. The protein combination responsible for the development of refractoriness is easily split off because the first precipitation of the sodium factor by means of solvents destroys the refractory power. Moreover the successful combination of desoxycorticosterone and protein in a relatively brief time following mixing, indicates that the combination is easily made. It is probable that the sodium factor normally exists as a haptene combination with a protein in ordinary adrenal extracts. Nitrogen is always present in such extracts.

The extreme specificity of the refractory state developed to desoxycorticosterone-adrenal protein is particularly significant. It is similar to the specificity observed immunologically which is developed to lipids. Immunological differences have been

demonstrated between cholesterol, pseudo-cholesterol and cholesterol esters which showed the importance of the presence and position of the —OH group (5). Cholesterol oxide and dibromide were non-antigenic (6).

The failure of refractoriness to develop to corticosterone or desoxycorticosterone is in agreement with immunological studies using lipids. FitzGerald (7) states that lipids will only act as true antigens when combined with protein.

In light of our present information a redefinition of the sodium factor should be made. The sodium factor is the substance in the adrenal responsible for sodium retention. It is probably associated or combined with a protein which accounts for the development of the refractory state.

SUMMARY

Precipitation of the sodium factor abolishes its power to confer refractoriness without diminishing its ability to cause sodium retention. Corticosterone and desoxycorticosterone act as precipitated sodium factor in being unable to confer refractoriness. Repeated injections of desoxycorticosterone plus pig serum led to the development of partial refractoriness. It was possible to develop complete refractoriness to a desoxycorticosterone-beef adrenal protein mixture. An animal made refractory to the latter was responsive to a corticosterone-beef adrenal protein mixture or to desoxycorticosterone-pig serum mixture.

We wish to thank Dr. Oliver Kamm of Parke, Davis and Company for adrenal glands, Dr. T. Reichstein for corticosterone and Dr. R. D. Shaner of Roche-Organon, Incorporated, for desoxycorticosterone.

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SODIUM CHLORIDE AND DEXTROSE APPETITE OF UNTREATED AND TREATED ADRENALECTOMIZED RATS

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IN PREVIOUS EXPERIMENTS it was found that adrenalectomized rats, when given free access to a variety of mineral solutions, selected sodium salts and by virtue of their increased sodium intake kept themselves alive and free from signs of insufficiency (1). Thus, by their appetite the adrenalectomized rats indicated that they needed sodium.

This appetite method has already been applied to the study not only of other glandular deficiencies but also of various vitamin deficiencies (2).

In the present experiments we have applied a similar technic in the study of the effects produced on the altered salt and carbohydrate metabolism of adrenalectomized rats by treatment with desoxycorticosterone acetate in oil,[1] adrenal cortex extract,[2] and charcoal adsorbate of adrenal cortical hormone.[3]

METHODS

The rats were kept in separate cages, approximately 10×8×13 in., each of which contained a food cup and three graduated inverted bottles—one filled with distilled water, one with a 3% solution of sodium chloride (C.P.), and one with a 40% solution of dextrose (U.S.P.X.). The food cup was filled with our stock diet made, however, without sodium chloride and with saltless butter. The diet contained the following substances: graham flour, 725 gm.; casein, 100 gm.; skim milk powder, 100 gm.; saltless butter, 50 gm.; calcium carbonate, 15 gm. Carbohydrate constituted 59.2% of the diet; fat, 14.1%; and protein, 26.7%. Throughout the experiment the same bottles were used, one each for sodium chloride, dextrose, and water; and each bottle remained in the same position in the cage.

The intake from each bottle and the food intake were recorded daily; body weight was recorded weekly. The bottles were emptied, cleaned, and refilled twice weekly.

In these experiments 25 rats (21 males and 4 females) were used. After 40 days, when the ages of the rats ranged from 85 to 206 days and the daily intake of fluids and food had reached constant levels, the adrenals were removed. Postoperatively the rats were observed for a preliminary period of 20 to 30 days. At this time 14 rats were killed and autopsied; of the remaining 11 rats, 6 were treated for 40 days with daily injections of desoxycorticosterone acetate and 5 for 48 days with daily injec-

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[1] Desoxycorticosterone acetate, in the form of Percorten, was supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J.

[2] The adrenal cortex extract was supplied by the Upjohn Company, Kalamazoo, Mich.

[3] The charcoal adsorbate of adrenal cortical hormone, in the form of Cortisorbate, was supplied by Schieffelin, & Co., New York City.

tions of cortical extract. Four of these rats (3 which had previously been treated with cortical extract and 1 with desoxycorticosterone acetate) were fed the stock diet with a high content of charcoal adsorbate of adrenal cortical hormone for 37 days.

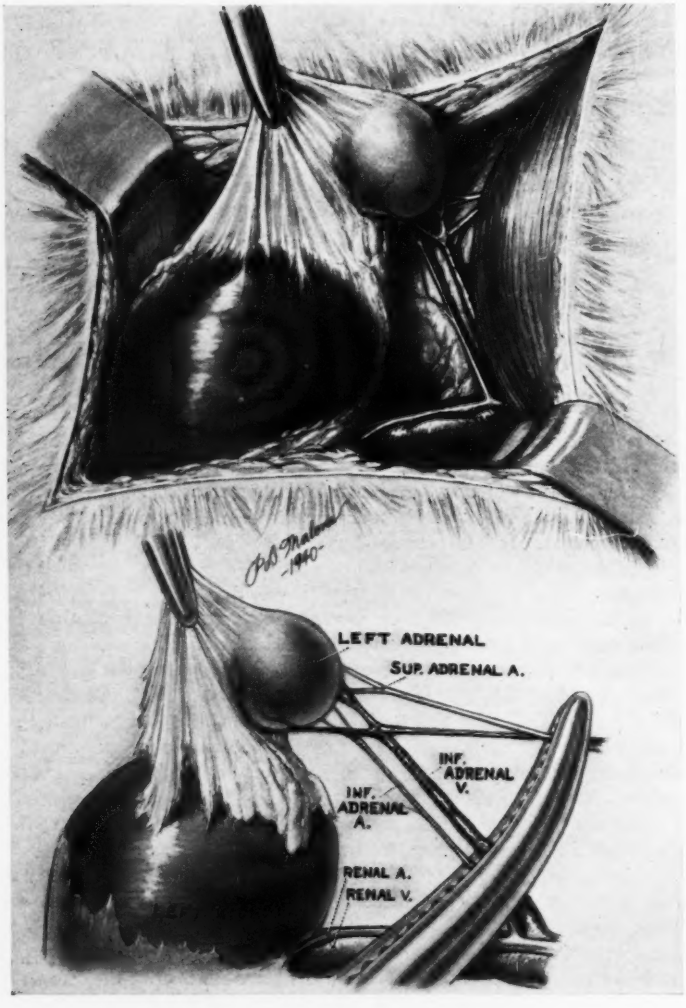


Fig. 1. Technic of adrenalectomy.

Figure 1 shows the operative methods used to remove the adrenals. Through an incision about 1 in. long, made parallel to the lowest rib, the kidney and the surrounding tissues were exposed. With a pair of mouse tooth forceps a hole was made in the capsule membrane near the posterior end of the kidney. The membrane was peeled away from the kidney toward the adrenals. Then the fat and the connective tissues

attached to the capsule were removed from the walls of the abdominal cavity, finally leaving the adrenal attached to the body only by a bundle of vessels and nerves. The greatest care was observed to avoid all manipulation of the adrenal in order to avoid all possibility of dislodging any cells. When the gland was thus freed and a clear vision was obtained of all the vessels, a curved hemostat was introduced deep into the opening and clamped on the inferior adrenal vein and artery as near as possible to the renal vein. The bundle of vessels was severed by running a scalpel over the upper edge of the hemostat. After a minute the hemostat was slowly released. Bleeding rarely resulted when the teeth of the hemostat fitted well and had previously been thoroughly cleaned and dipped in water or salt solution to prevent the vessels from sticking to the metal.

The success of our operative technic was determined by two different methods. For the first method the rats were kept on sodium chloride free food without access

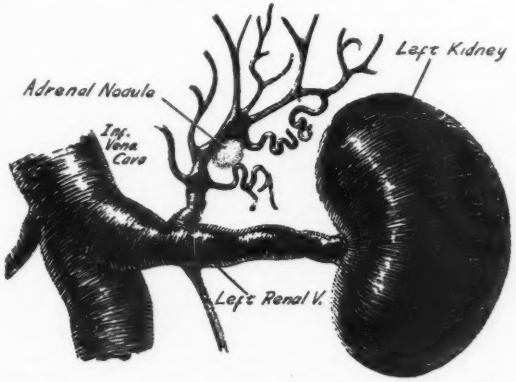


Fig. 2. PROLIFIC BRANCHING OF THE VESSELS OF THE INFERIOR ADRENAL VEIN AND THE LOCATION OF A NODULE OF ADRENAL TISSUE IN AN ANGLE MADE BY ONE OF THE BRANCHES AND THE MAIN VEIN.

to sodium chloride solution. With our operative technic we now obtain a 100% mortality with such a dietary regime. Formerly occasional rats survived. In such rats small pieces of adrenal tissue were found to have remained due to accidental rupture of the adrenal capsule. The second method gave even a better test for the completeness of the operation. The rats were kept on a sodium chloride free diet but given access to sodium chloride solution. With this technic we now obtain a 100% survival, and 100% of the animals manifest a definitely increased intake of sodium chloride solution. Formerly, in instances in which the rats did not increase their sodium chloride solution intake within the first few days after operation, we found that discontinuation of the access to sodium chloride after 15 to 20 days did not kill the rats or have any noticeable effect on them. At autopsy such animals always showed one or several small nodules of adrenal tissue in the forks of the newly sprouted branches of the inferior adrenal vein on the left side (fig. 2). We have never found such nodules on the right. These nodules remained apparently because the hemostat had not been applied on the adrenal vessels sufficiently close to the renal vein. When rats are kept on diets which contain the ordinary amount of sodium chloride, it is very difficult to know whether the adrenalectomy has been complete or not, since about 60% of the rats survive and since no adequate tests are available to determine the completeness of the operation.

RESULTS

Sodium Chloride and Dextrose Appetite of Untreated Rats

Figure 3 gives the sodium chloride (3%), dextrose (40%), and water intake of one of the 14 untreated rats for 18 days before adrenalectomy and for 20 days afterwards.

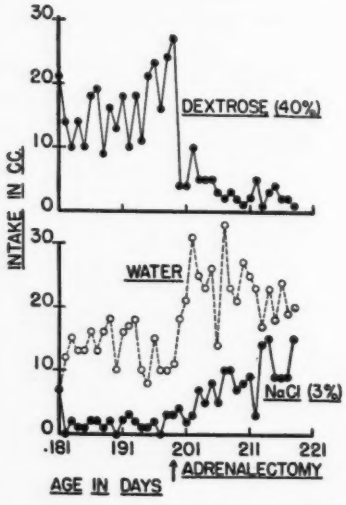


Fig. 3. TYPICAL EFFECTS PRODUCED ON THE SODIUM CHLORIDE AND DEXTROSE APPETITE IN A RAT BY ADRENALECTOMY. Loss of fluid in evaporation has not been deducted.

Shortly after adrenalectomy the dextrose intake dropped sharply from an average of 15.1 cc. per day to 2.3 cc.; the average water intake increased from 12.0 cc. per day to 20.5 cc.; the sodium chloride intake increased steadily from an average of 0.4 cc. to 8.6 cc.

Table 1 summarizes the results of the observations made on the 14 rats. It gives the average daily intake for the 3 fluids and the stock diet for 10 days before adrenalectomy and for the 10 days between the 10- and 20-day period after adrenalectomy. All 14 rats showed an increased appetite for sodium chloride and a decreased appetite for dextrose. The average daily sodium chloride solution intake increased from 1.1 to 9.5 cc.; simultaneously, the average daily dextrose solution intake decreased from 17.3 to 5.8 cc. Water intake increased from an average daily intake of 10.4 cc. to 19.6 cc. The total water intake, including the water in the dextrose and sodium chloride solutions, increased from 21.9 to 32.6 cc. In this and in the 3 other tables 1.2 cc. per day was deducted from the sodium chloride and water records for evaporation.

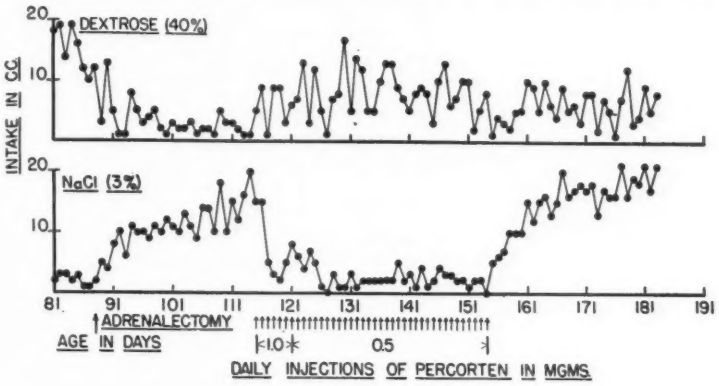
That the decreased dextrose intake represented a specific change in carbohydrate appetite is shown by the fact that after adrenalectomy the intake of the stock diet showed only a small change. The daily food intake was recorded for 5 rats. In 4 rats the intake either remained the same or increased; in 1 rat it decreased. The daily food intake increased from 8.5 gm. per day for the 10 days before adrenalectomy to 9.7 gm. in the 10- to 20-day period afterwards.

It will be noted that the daily intake of stock food before adrenalectomy averaged 8.5 gm., which is about 6 gm. less than the average for normal rats not given access to

dextrose. However, measured in calories the decrease in food intake was almost exactly balanced by the dextrose intake.

Sodium Chloride and Dextrose Appetite of Treated Rats

Desoxycorticosterone acetate (*Percorten*). Figure 4 gives a typical record of the sodium chloride and dextrose intake of one of the 6 adrenalectomized rats treated with desoxycorticosterone acetate. This rat was placed in the experimental cage at



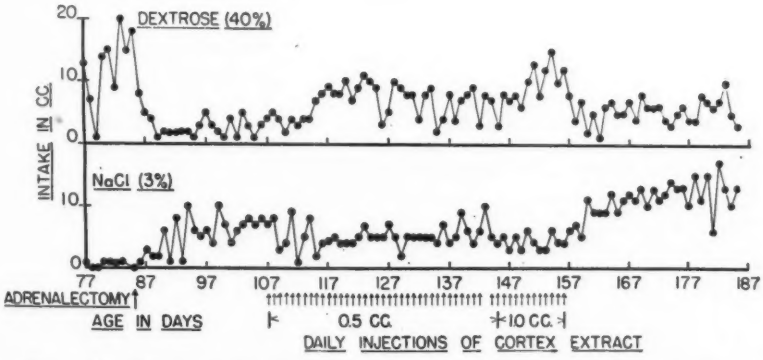


Fig. 4. (above) Typical effects produced on the sodium chloride and dextrose appetite in a rat by daily desoxycorticosterone acetate injections. Fig. 5 (below). Typical effects produced on the sodium chloride and dextrose appetite in a rat by daily injections of cortical extract.

the age of 81 days, adrenalectomized at 88 days, and at 115 days started on daily injections of desoxycorticosterone acetate for 40 days (1.0 mg. daily for 7 days and 0.5 mg. for 33 days). Adrenalectomy had sharply decreased the average daily dextrose intake from 15.4 to 2.3 cc. and increased the sodium chloride appetite from 0.9 to 10.3 cc. Desoxycorticosterone acetate treatment produced an almost immediate increase in the dextrose intake and a decrease in the sodium chloride intake. At the end of the 40 days the dextrose intake had increased to 8.5 cc., slightly more than half as high as its preoperative level of 15.4 cc.; but the sodium chloride intake had returned to 1.0 cc. (preoperative level was 0.9 cc.). Discontinuation of treatment was followed by only a small decrease in the dextrose intake from 8.5 to 6.1 cc. per day, while the

TABLE 1. AVERAGE DAILY INTAKE 10 DAYS BEFORE AND 10 TO 20 DAYS AFTER ADRENALECTOMY

Rat No.	Sex	Age at adrenalectomy	Sodium chloride, 3%		Dextrose, 40%		Water		Food (McCollum)	
			Before	After	Before	After	Before	After	Before	After
		days	cc.	cc.	cc.	cc.	cc.	cc.	gm.	gm.
1	♂	199	1.5	8.0	19.5	6.9	9.9	20.7		
2	♂	199	0.8	5.9	17.9	4.6	15.4	27.3		
3	♂	199	0.4	8.6	15.1	2.3	12.0	20.5		
4	♂	196	2.5	11.7	28.5	10.7	3.9	9.3		
5	♂	196	2.9	12.1	22.6	4.7	12.4	20.2		
6	♂	190	0.1	9.2	15.6	6.0	7.0	24.8		
7	♂	196	0.4	9.7	2.8	0.9	15.0	23.3		
8	♂	206	0.0	8.6	25.8	7.7	6.0	12.1		
9	♂	190	0.6	12.3	19.3	6.7	8.9	21.2		
10	♂	130	0.2	10.8	9.6	3.3	16.5	24.3	10.9	16.4
11	♀	94	2.2	6.3	6.0	4.5	14.0	18.1	10.6	7.7
12	♀	95	0.5	7.8	21.4	9.7	5.5	13.3	6.1	6.7
13	♀	95	0.0	11.0	20.4	5.2	8.0	23.1	6.6	9.5
14	♀	95	3.3	10.6	18.3	7.5	10.4	16.4	8.2	8.2
Average		164	1.1	9.5	17.3	5.8	10.4	19.6	8.5	9.7

sodium chloride intake increased within the first 24 hours and so sharply that after 30 days it had surpassed its pretreatment level (17.0 as compared to 10.3 cc.). At the age of 182 days the rat weighed 305 gm., which falls within the normal range of weights for rats of this age; and it appeared to be in good health and free from signs of insufficiency.

TABLE 2. DESOXYCORTICOSTERONE ACETATE (40 DAYS—1.0 MG. DAILY FOR 7 DAYS, 0.5 MG. FOR 33 DAYS)

Rat No.	Sex	Age at adrenalectomy	Average daily intake of sodium chloride (3% solution)				Average daily intake of dextrose (40% solution)			
			10 days before adrenalectomy	10–20 days after adrenalectomy	Last 10 days of treatment	20–30 days after last treatment	10 days before adrenalectomy	10–20 days after adrenalectomy	Last 10 days of treatment	20–30 days after last treatment
		days	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
15	♂	88	0.4	7.5	0.6	14.0	12.4	1.8	4.7	3.4
16	♂	88	0.5	11.0	2.4	20.1	11.7	3.6	9.7	8.3
17	♂	88	0.9	10.3	1.0	17.0	15.4	2.3	8.5	6.1
18	♂	86	0.0	5.5	0.2	10.8	9.7	3.9	8.3	6.1
19	♂	88	0.8	8.7	2.5	23.2	18.1	2.8	10.2	12.7
20	♂	86	0.5	6.7	1.7	12.9	11.3	2.8	5.3	2.7
Average		87	0.5	8.3	1.4	16.3	13.1	2.9	7.8	6.6
			Average daily water intake				Average daily food intake			
							gm.	gm.	gm.	gm.
15	♂		11.5	15.8	14.7	34.7	8.5	5.7	12.8	10.9
16	♂		11.4	21.6	11.3	33.9	9.8	7.5	11.3	10.8
17	♂		7.2	17.9	7.8	22.0	7.5	6.0	11.7	11.8
18	♂		11.6	15.9	9.3	20.6	8.8	7.2	10.8	10.8
19	♂		3.8	16.4	11.2	17.3	5.5	4.7	12.1	10.3
20	♂		8.8	13.8	12.4	21.8	8.8	7.3	12.7	11.1
Average			9.1	16.9	11.1	25.1	8.2	6.4	11.9	11.0

Table 2 summarizes the results of the observations made on the 6 rats treated with desoxycorticosterone acetate. The average daily intake of sodium chloride solution increased from 0.5 cc. for the 10 days before adrenalectomy to 8.3 cc. for the 10- to 20-day period after adrenalectomy, decreased again to 1.4 cc. for the last 10 days of treatment, and increased to 16.3 cc. between the 20th and 30th day after the discontinuation of the treatment. All 6 rats showed a markedly increased sodium chloride appetite after adrenalectomy, a decrease almost to normal under treatment, and a return after treatment was stopped. With larger doses of desoxycorticosterone acetate the intake during the treatment period undoubtedly would have decreased to an even lower level. After discontinuation of the treatment the intake increased to almost twice the level present before treatment was started (16.3 cc. as compared to 8.3 cc.). The significance of this finding is not clear. It may be that after a period of treatment the adrenalectomized animals become more dependent than before on sodium chloride.

The average daily intake of dextrose solution decreased from 13.1 cc. for the 10 days preceding adrenalectomy to 2.9 cc. for the 10- to 20-day period afterwards, increased to 7.8 cc. during the last 10 days of treatment, and decreased again to 6.6 cc. from the 20th to 30th day after the last injection. All 6 rats showed this distinct decrease in dextrose appetite after adrenalectomy and an increase under treatment, and 5 of the 6 animals showed a decrease again after the injections were stopped. However, although under the desoxycorticosterone acetate treatment the dextrose intake showed a definite increase (2.9 to 7.8 cc.), it still fell short of the original preoperative level of 13.1 cc. Here again, just as with the sodium chloride appetite, it is possible that a higher dosage of desoxycorticosterone acetate would have produced a more marked effect. After discontinuation of the treatment the dextrose appetite decreased only slightly (7.8 to 6.6 cc.). The significance of this failure of the dextrose appetite to return to the low pretreatment level of 2.9 cc. we are unable to determine. It may depend in some way on the increased sodium chloride appetite.

Water intake also showed marked changes. The average daily intake increased from 9.1 to 16.9 cc. after adrenalectomy, decreased to 11.1 cc. under treatment, and increased again to 25.1 cc. after treatment. The total water intake for the sodium chloride, dextrose, and water increased from 17.5 cc. to 26.9 cc. after adrenalectomy, decreased to 17.2 cc. under treatment, and increased to 45.2 cc. after treatment. The total water intake closely paralleled the sodium chloride intake. Food intake decreased slightly in most of the animals. It averaged 8.2 gm. before and 6.4 gm. after adrenalectomy. Under treatment it increased to 11.9 gm. and after treatment decreased very slightly to 11.0 gm.

Cortical extract. Figure 5 gives the sodium chloride and dextrose intake of one of the 5 adrenalectomized rats treated with cortical extract. Adrenalectomy increased the sodium chloride from 0.0 to 5.2 cc. and decreased the dextrose intake from 11.7 to 2.8 cc. Daily injections of cortical extract in doses of 0.5 to 1.0 cc. decreased the sodium chloride solution intake to 3.0 cc. and increased the dextrose intake to 9.7 cc. Discontinuation of the treatment was followed by a sharp increase in sodium chloride intake and a decrease in dextrose intake.

Table 3 summarizes the results of the observations made on the 5 rats. The average daily intake of sodium chloride solution increased from 0.3 cc. for the 10 days before adrenalectomy to 7.3 cc. 10 to 20 days afterwards. Daily injections of 0.5 cc. per day for 36 days and 1.0 cc. for 12 days decreased the sodium chloride solution intake to 3.0 cc.; 20 to 30 days after discontinuation of the treatment it had increased again to 12.4 cc. Without exception the sodium chloride appetite of the 5 rats increased after adrenalectomy, decreased under cortical extract treatment, and increased again after discontinuation of treatment. It will be noted, however, that under treatment

TABLE 3. CORTICAL EXTRACT (48 DAYS—0.5 CC. DAILY FOR 36 DAYS, 1.0 CC. FOR 12 DAYS)

Rat No.	Sex	Age at adrenalectomy	Average daily intake of sodium chloride (3% solution)				Average daily intake of dextrose (40% solution)			
			10 days before adrenalectomy	10–20 days after adrenalectomy	Last 10 days of treatment	20–30 days after treatment	10 days before adrenalectomy	10–20 days after adrenalectomy	Last 10 days of treatment	20–30 days after treatment
		days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
21	♂	85	0.0	5.2	3.0	11.1	11.7	2.8	9.7	6.0
22	♂	93	0.2	7.3	2.7	13.1	5.0	2.1	4.5	4.7
23	♂	94	0.4	6.5	3.1	6.3	13.3	3.4	12.4	10.1
24	♂	93	0.7	8.3	3.7	15.5	12.7	9.5	20.1	6.6
25	♂	86	0.3	9.0	2.4	16.0	11.1	4.2	8.5	6.0
Average		90	0.3	7.3	3.0	12.4	10.8	4.4	11.0	6.7
			Average daily water intake				Average daily food intake			
							gm.	*gm.*	*gm.*	*gm.*
21	♂		10.9	17.4	9.2	16.2	8.1	8.2	9.8	9.3
22	♂		17.7	22.4	17.7	26.7	9.1	10.1	11.6	11.9
23	♂		6.4	14.4	12.4	14.9	6.8	9.0	11.5	14.3
24	♂		12.5	20.5	8.6	23.7	7.6	6.6	7.8	10.2
25	♂		21.1	18.5	16.6	21.4	8.6	9.2	11.7	12.6
Average			13.7	18.6	12.9	20.6	8.0	8.6	10.5	11.7

the sodium chloride appetite did not decrease as much as with desoxycorticosterone acetate. Under the cortical extract treatment the rats still took a fairly large amount of the sodium chloride solution (3.0 cc. as compared to the preoperative level of 0.3 cc.). After discontinuation of the treatment the sodium chloride appetite became greater than it had been before treatment (the average daily intake was 12.4 as compared to 7.3 cc.) just as it did after discontinuation of the desoxycorticosterone acetate treatment.

The average daily dextrose intake decreased from 10.8 cc. for the 10 days before adrenalectomy to 4.4 cc. for the 10 to 20 days afterwards, increased to 11.0 cc. during the last 10 days of treatment with cortical extract, and decreased again to 6.7 cc. for the 20 to 30 days after discontinuation of the treatment.

After adrenalectomy all 5 rats showed this decreased dextrose appetite which increased in every case under treatment. Furthermore, a comparison of the intake of the individual animals shows that in direct contrast with desoxycorticosterone acetate the injection of the cortical extract had a greater effect on the dextrose appetite than on the sodium chloride appetite. However, after discontinuation of the treatment the dextrose appetite showed a much smaller change (11.0 to 6.7 cc. as compared to 3.0 to 12.4 cc. for sodium chloride intake). Here again the failure of the dextrose appetite to return to a lower level may depend on the greatly heightened sodium chloride appetite.

Cortisorbate. Four adrenalectomized rats which had been used for the desoxycorticosterone acetate or cortical extract experiments were put on a diet with a high content of charcoal adsorbate of adrenal cortical hormone (Cortisorbate) (75 gm. to 150 gm. per 1000 gm. of food) for 37 days; this represented an average daily intake of 1.7 gm. of charcoal adsorbate for the last 10 days of treatment. At the time they were placed on this diet, an average of 46 days after discontinuation of the other treat-

TABLE 4. CHARCOAL ADSORBATE FOOD (37 DAYS—75 GM. PER 1000 GM. OF FOOD DAILY FOR 23 DAYS, 150 GM. PER 1000 GM. OF FOOD FOR 14 DAYS)

Rat No.	Sex	Age at adrenalectomy	Average daily intake of sodium chloride (3% solution)			Average daily intake of dextrose (40% solution)			Average daily intake of water			Average daily intake of food			Average daily intake of charcoal adsorbate
			10 days before adrenalectomy	Last 10 days before treatment	Last 10 days of treatment	10 days before adrenalectomy	Last 10 days before treatment	Last 10 days of treatment							Last 10 days of treatment
		days	cc.	cc.	cc.	cc.	cc.	cc.							gm.
17	♂	88	0.9	19.7	10.1	15.4	7.0	13.3	7.2	30.0	21.4	7.5	9.8	10.9	1.6
22	♂	93	0.2	13.5	4.3	5.0	8.2	12.6	17.7	25.9	28.0	9.1	12.6	11.3	1.7
24	♂	93	0.7	16.5	6.4	12.7	6.6	11.3	12.5	26.5	17.7	7.6	10.7	9.6	1.4
25	♂	86	0.3	15.1	9.9	11.1	10.4	15.1	21.1	20.4	20.7	8.6	13.6	12.6	1.9
Average		90	0.5	16.2	7.7	11.1	8.1	13.1	14.6	25.7	22.0	8.2	11.7	11.1	1.7

ments, the sodium chloride and dextrose intake had reached fairly constant levels. Table 4 summarizes the results. The sodium chloride intake, which had increased from 0.5 cc. for the last 10 days before adrenalectomy to 16.2 cc. for the last 10 days before charcoal adsorbate treatment, decreased to 7.7 cc. for the last 10 days of treatment. Dextrose intake decreased from 11.1 cc. before to 8.1 cc. after adrenalectomy and increased to 13.1 cc. during the last 10 days of treatment. Water intake increased from 14.6 to 25.7 cc. after adrenalectomy and decreased slightly under treatment. Food intake increased from 8.2 to 11.7 gm. after adrenalectomy and remained almost the same during treatment.

DISCUSSION

These experiments show that by their appetites the adrenalectomized rats indicated that they needed more sodium chloride and less carbohydrate than normal animals. Their need for sodium chloride has been well established by biochemical total exchange studies. From evidence obtained from biochemical studies, it would seem most likely that the decreased appetite for dextrose indicates an inability to absorb carbohydrate rather than an inability to utilize it (3).

According to the rats' appetites, desoxycorticosterone acetate affects both the sodium chloride and carbohydrate balance. However, it has a more marked effect on the sodium chloride than on the carbohydrate metabolism. Cortical extract also must affect both, but the metabolism of sodium chloride is much less improved than that of carbohydrate. These results agree fairly closely with the clinical and biochemical

observations made with these hormones. The charcoal adsorbate had a greater effect on sodium chloride appetite than on dextrose appetite.

The results of these experiments do not permit any definite conclusion to be drawn regarding the relative potency of these three preparations. We may, however, attempt a rough comparison on the basis of the effects produced on the sodium chloride and dextrose appetites. Desoxycorticosterone acetate in a daily dosage of 0.5 mg. reduced the sodium chloride appetite to a very low level. It is not unlikely that half this dosage, or even less, would have produced an almost equally large effect. Cortical extract in a daily dosage of 1.0 cc. reduced the sodium chloride appetite to a level which was still about twice as high as that found in the desoxycorticosterone acetate treated rats. Thus, about twice this dosage, or 2.0 cc., might have had about the same effect as 0.5 mg. of desoxycorticosterone acetate. Similarly, charcoal adsorbate in a daily dosage of 1.7 gm. had about half the effect of the 1.0 cc. dosage of cortical extract or one-quarter the effect of the 0.5 mg. dosage of desoxycorticosterone acetate. Thus, 0.5 mg. of desoxycorticosterone acetate would be roughly equivalent to 2.0 cc. of cortical extract or to 6.8 gm. of charcoal adsorbate. This agrees closely with the clinical results obtained with a different technic by Dr. George Thorn (personal communication). Measured in terms of fresh gland substance a comparison can be made between cortical extract and charcoal adsorbate. The preparation of 1.0 cc. of cortical extract required 50 gm. of fresh gland substance; the preparation of 1.0 gm. of charcoal adsorbate, 10 gm. Accordingly, on the basis of the equated values determined above, 2.0 cc. of cortical extract, or 100 gm. of fresh gland substance, would be required to produce the same effect as 6.8 gm. of charcoal adsorbate, or 68 gm. of fresh gland substance. The charcoal adsorbate would thus appear to have a somewhat higher potency. For practical purposes charcoal adsorbate would have only a limited value since the 6.8 gm. required to reduce the sodium chloride intake to its normal level would constitute a third to a half of the entire food intake. When comparing these values with those derived by other methods, it is important to keep in mind that the basal food of these rats was practically completely devoid of sodium chloride.

It is interesting that 2 patients with Addison's disease whose salt appetite was examined in some detail had a marked aversion to sweets. One of the patients was a 3-year-old boy who ate salt in extremely large amounts. This child would not touch sweets or milk (4). A 34-year-old patient with Addison's disease, examined last year, who ate salt in enormous amounts, even on oranges, lemons and grapefruit, also had a strong aversion to sugar and candy. These may be isolated instances. We have not had an opportunity to examine a larger number of patients with Addison's disease. The removal of the adrenal medulla along with the cortex may, of course, account for any differences in appetite which exist between the adrenalectomized rats and the patients suffering from Addison's disease.

SUMMARY

Adrenalectomized rats kept on a sodium chloride free diet manifested an increased appetite for sodium chloride and a decreased appetite for the carbohydrate (dextrose). All of the rats survived and remained free from signs of insufficiency.

Their water intake showed a marked increase, while the intake of the stock diet either remained constant or actually increased.

Daily treatment of 6 rats with desoxycorticosterone acetate decreased the sodium chloride appetite essentially to its normal level and increased the dextrose appetite, however, not to the normal level. Discontinuation of the treatment reversed the

appetite again. The desoxycorticosterone had a marked effect on the appetite for both substances, but more on the sodium chloride than on the dextrose appetite.

Daily treatment with cortical extract in 5 rats also decreased the appetite for sodium chloride and increased the appetite for dextrose, while discontinuation of the treatment caused a reversal of the appetite. Compared to the desoxycorticosterone acetate, it had markedly less effect on the sodium chloride appetite, but approximately the same effect on the dextrose appetite.

Total water intake decreased sharply during treatment, more with desoxycorticosterone acetate than with the cortical extract, and increased sharply after discontinuation of treatment. It seemed to follow closely the intake of sodium chloride.

Charcoal adsorbate of adrenal cortical hormone given to 4 rats decreased the sodium chloride and increased the dextrose appetite. However, the magnitude of the changes was small considering the large amounts of the charcoal adsorbate which the rats received.

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DISTRIBUTION OF ADRENO-CORTICAL ('AC') HORMONES IN TISSUES OF THE RAT UNDER NORMAL AND EXPERIMENTAL CONDITIONS[1]

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THE SYNTHETIC FORMATION of compounds consisting of adrenalin combined with hormonal sterols, and the presence and chemical determination of such adreno-cortical ('AC') compounds in the blood as natural products of suprarenal secretion have been discussed in a previous paper (1).

The following experiments with tissues were carried out by means of a modification of Shaw's method (2) originally designed for the determination of 'adrenalin.' Only those technical details will be given below which differ from the modification as described for AC determination in blood (1).

METHOD

Four hundred to eight hundred mg. of fresh tissue are washed in physiological saline solution, superficially dried between filter papers and weighed. Then the tissue is placed in a small porcelain mortar containing about 2 cc. of 10% trichloracetic acid and cut finely with scissors. One small teaspoonful (about 5 gm.) of quartz sand (Ottawa) is added and the tissue and sand are very thoroughly ground with a pestle until a fine brei is formed. Particles adhering to the pestle are rinsed into the mortar with more trichloracetic acid and the entire contents of the mortar are rinsed upon a filter paper (9 cm. diameter) which has been previously soaked with trichloracetic acid and placed in a glass funnel. The latter is inserted in a small graduated glass cylinder. The residue on the filter is washed with trichloracetic acid until the volume of filtrate is about 12 cc. The filtrate is then transferred into a small beaker (50 cc.) two drops of a fresh aqueous phenolphthalein solution (1:1000) are added and 4% NaOH is added from a burette until a pink color developes. The pH is then adjusted carefully to 4.0 by adding N/5 H_2SO_4. (A Beckman pH meter was used.) The total fluid is divided into 2 exactly equal parts in 2 graduated 15 cc. centrifuge tubes. Two cc. of $Al(OH)_3$ suspension are added to each tube and the further procedure is carried out in the same manner as described in the previous paper (1) beginning from step 5.

For determination of the so-called specificity ratio (sp.r.) which allows a rough estimate of the relative concentration of adrenalin within the bulk of AC compounds, the contents of one tube are worked up in the ordinary way, while to the second tube about 0.05 cc. of N/1 H_2SO_4 is added in step 9 of the procedure instead of the 0.35 cc. of 4% NaOH (1).

The figures read from the standard curve after colorimetric comparison with the adrenalin standard solution are multiplied by 4000[2] and divided by the weight

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[1] This study was aided by a grant from the Rockefeller Foundation.
[2] It is necessary to multiply the readings from the curve first by 2 because the curve was constructed in using 2 cc. of the 10^{-7} adrenalin standard solution for comparison with 2 cc. of blood, then by 2 because the result is to be divided by the total weight of tissue taken, while only one-half of this amount was used for each of the two determinations which are necessary to find the specificity ratio, and further multiply by 1000 because the result is to be expressed in grams.

in milligrams of fresh tissue used. The results indicate the AC concentration in the tissue, expressed in color units per gram (each unit corresponding to the color intensity of 1 mγ/gm. of adrenalin).

The specificity ratio (sp.r.) is determined by dividing the 'alkaline' result by the 'acid' result (sp.r. = 1:alk./ac.). A quotient alk./ac. greater than 1.0 is suggestive of the presence of relatively large amounts of adrenalin in comparison with the sterol component and with a certain amount of ascorbic acid which is also represented in the figures obtained with this method. A quotient of 3.5 which is characteristic of pure 'free' adrenalin, or still larger quotients, are never found in blood and in tissues, except for muscles where they are not infrequently encountered under certain experimental conditions.

With the exception of two groups of gray male rats, white male albino rats of the same stock (age 2 to 3 months, weight 150 to 200 gm.) were used in all experiments. All animals were fed with Purina Dog Chow Checkers.

The hearts were examined in all instances but these findings will be published in a separate study. The findings discussed below concern the tissues of muscles of the thigh, of the decapsulated right kidney, of one brain hemisphere, of the right lobe of the liver and of the spleen.

TABLE 1

Rat No.	Date	Kidney		Liver		Rat No.	Date	Brain		Spleen		Muscle	
		C.U.[1]	sp.r.[2] 1:	C.U.	sp.r. 1:			C.U.	sp.r. 1:	C.U.	sp.r. 1:	C.U.	sp.r. 1:
Summer Standard Values													
1	7/26	1229	1.37	766	1.06	11	8/12	1031	0.79	1822	1.00	110	1.20
2	7/26	240	1.00	136	1.00	12	8/13	1739	0.87	2276	0.87	750	3.20
3	7/29	911	1.23	1933	1.00	13	8/13	1170	0.62	1471	0.95	871	2.61
4	7/30	1349	1.32	1510	1.06	14	8/14	1488	0.84	1919	1.14	397	2.60
5	7/31	1032	—	661	1.09	15	8/15	1407	0.69	1454	1.01	525	1.56
6	8/1	1419	0.83	1301	0.83	16	8/18	858	0.83	2457	1.08	710	—
7	8/2	1206	1.05	1437	0.85	17	8/22	1473	1.09	2292	1.00	1062	1.47
8	8/3	1540	1.01	1617	0.89	18	8/23	1270	1.06	1373	0.89	773	0.90
9	8/5	1278	1.09	1348	0.79	19	8/23	1132	0.85	1814	1.16	1014	1.05
10	8/6	1714	1.12	1645	1.07	20	8/24	1360	0.88	1025	1.02	816	1.26
Average		1192	1.11	1335	0.96			1293	0.85	1790	1.01	703	1.77
Winter Standard Values													
21	12/5	1693	1.11	1896	0.98	31	1/13	2257	1.00	4617	1.26	1617	1.24
22	12/5	1067	1.44	3074	1.00	32	1/13	2465	0.90	3884	1.05	1232	1.52
23	12/7	1414	0.95	2605	1.03	33	1/15	2408	1.00	1402	0.70	1167	1.95
24	12/7	1149	1.08	1983	0.97	34	1/15	3018	0.81	3277	1.03	1557	2.75
25	12/9	1605	1.00	1408	1.14	35	1/16	2916	0.96	2714	1.08	1426	1.72
26	12/9	1265	1.06	1412	1.00	36	1/16	2336	0.94	2963	1.03	878	2.45
27	1/30	1832	1.18	1676	1.25	37	1/31	1608	0.86	1911	1.00	414	1.40
28	1/31	1221	1.11	2056	1.20	38	2/1	1582	—	2356	1.02	781	2.45
29	2/1	1045	0.95	1646	0.90	39	2/1	1776	0.94	1912	0.80	552	2.50
30	2/2	1477	0.95	2293	1.00	40	2/2	2191	0.93	3511	1.03	1115	1.93
Average		1377	1.08	2005	1.05			2256	0.93	2855	1.00	1074	1.99

[1] C.U., color units per gm. of tissue, one U equalling the color effect of one billionth of a gm. of adrenalin.

[2] sp.r., specific ratio, depending on the qualitative composition of the AC compounds. The larger the figures in this column are (above 1.00) the more adrenalin can be assumed to be present in comparison with the cortical sterols participating in the AC compounds.

TABLE 2. GREY RATS

Standard values (summer)						Injection of adrenalin[1]					
Rat No.	Date	Kidney		Liver		Rat No.	Date	Kidney		Liver	
		c.u.	sp.r. 1:	c.u.	sp.r. 1:			c.u.	sp.r. 1:	c.u.	sp.r. 1:
67	7/10	1073	0.98	—	—	75	7/15	1488	1.08	2602	1.16
68	7/10	1269	1.00	—	—	76	7/16	1500	1.03	2792	1.15
69	7/11	1433	0.90	2092	1.00	77	7/16	1590	1.39	2414	1.18
70	7/11	1374	1.00	2117	1.00	78	8/7	2182	1.66	1840	—
71	7/12	1755	1.32	3097	0.93	79	8/8	1491	1.00	1824	0.90
72	7/12	1029	1.03	1366	0.87	80	8/8	1673	1.21	1829	1.00
73	8/7	2121	1.13	1921	0.86	81	8/9	1736	1.22	2683	1.00
74	8/8	1562	0.95	1909	0.83	82	8/9	1760	1.29	2327	1.10
—	—	—	—	—	—	83	8/10	2846	1.31	2104	0.91
—	—	—	—	—	—	84	8/10	1693	1.01	2592	1.00
Average		1451	1.04	2083	0.91	Average		1796	1.22	2301	1.04
White rat standard		1192	1.11	1335	0.96	Gray rat standard		1451	1.04	2083	0.91
Deviation		+22%		+56%		Deviation		+24%		+11%	

[1] *Rats* 75–77 had received 2.5γ adrenalin per gm. body weight, *rats* 78–84 had received 5.0γ per gm. The *rats* 75 and 80–84 died spontaneously after intervals from 5 to 12 minutes due to vigorous massage of the place where adrenalin had been injected. This procedure which was avoided in all other animals tended to speed up the effect of the injected adrenalin. *Rats* 76–79 were killed after 20 minutes.

Individual, Seasonal and Racial Variations

In white male rats individual variations both of the total AC figures and of the specificity ratios were considerable but with few exceptions the results obtained in each type of tissue and in each group of experiments were of an order sufficiently consistent to yield characteristic average figures. The range of individual differences both of the total AC values and of their qualitative composition, as reflected by the specificity ratios (sp.r.), are presented in the tables.

The lowest AC concentration was found in muscle tissue (only the heart showed still lower concentrations) while its sp.r. of 1.77 indicated a relatively high proportion of 'free' adrenalin within the bulk of AC in the muscle (table 1).

Higher AC concentrations were present in other organs (table 1) increasing in the following order: kidney, brain, liver and spleen, all of these containing only a little or no 'free' adrenalin. The sp.r. of the brain (1:0.85) suggested a comparatively high concentration of ascorbic acid, the sp.r. of which is 1:0.3. This interpretation is supported by observations of Hawley, Daggs and Stephens (3) who also found a higher concentration of ascorbic acid in the brain than in kidney, liver and spleen.

The above-mentioned findings were recorded during the summer months. Values of the same order were found occasionally until late in October. However, during the winter months a seemingly abrupt and marked elevation of the AC concentrations in the tissues took place which made it necessary to determine special 'winter standards' for purposes of experimental comparison. The relationship between summer and winter standard results is evident from table 1. The average sp.r.'s of each organ remained almost unchanged throughout the seasons.

Considering the average amounts of ascorbic acid in various organs as given by Hawley, Daggs and Stephens (3) and considering the color intensity of ascorbic acid as being 1/320th of that of adrenalin (1), ascorbic acid can be estimated to form

TABLE 3

Injection of adrenalin[1]														Injection of desoxycorticosterone acet.[2]					
Rat No.	Date	Kidney		Liver		Rat No.	Date	Brain		Spleen		Muscle		Rat No.	Date	Kidney		Liver	
		c.u.	sp.r. 1:	c.u.	sp.r. 1:			c.u.	sp.r. 1:	c.u.	sp.r. 1:	c.u.	sp.r. 1:			c.u.	spr. 1:	c.u.	sp.r. 1:
41	7/25	1535	1.00	1373	1.05	51	8/12	1016	1.03	1719	1.04	798	1.50	57	8/15	1859	1.04	1571	0.91
42	7/25	1684	1.22	1205	1.22	52	8/13	943	1.36	1483	1.00	988	1.92	58	8/16	1489	0.66	1805	1.11
43	7/29	1790	1.08	2348	1.06	53	8/14	1509	0.80	887	0.98	1034	2.55	59	8/17	1269	0.95	1883	1.10
44	7/30	1900	—	1690	1.21	54	8/14	1933	0.97	2666	1.04	752	2.00	60	8/19	1399	1.22	1228	—
45	7/31	611	1.49	1142	1.10	55	8/17	1373	0.87	1119	—	602	0.90	61	8/20	1813	1.25	2265	0.97
46	8/1	1729	1.34	1917	1.15	56	8/20	2201	0.98	1999	—	858	1.54	62	8/22	2103	1.15	2616	1.09
47	8/2	1610	1.08	1326	1.11	—	—	—	—	—	—	—	—	63	8/23	1411	0.91	1283	1.08
48	8/3	1506	1.13	1655	1.18	—	—	—	—	—	—	—	—	64	8/24	1322	1.08	1557	0.88
49	8/5	1046	1.44	1713	1.19	—	—	—	—	—	—	—	—	65	8/24	1589	1.09	2747	0.96
50	8/6	1840	1.54	1319	1.08	—	—	—	—	—	—	—	—	66	8/26	1613	1.12	2284	1.33
Average		1517	1.26	1569	1.14			1496	1.00	1646	1.01	839	1.74	Average		1587	1.05	1924	1.05
Summer standard		1192	1.11	1335	0.96			1293	0.85	1790	1.01	703	1.77	Summer Standard		1192	1.11	1335	0.96
Deviation		+28%		+18%				+16%		−8%		+19%		Deviation		+33%		+44%	

[1] All animals in this group received 2.5γ of adrenalin per gm. body weight and were killed after 20 minutes, except 47 and 53 which died spontaneously after 12 and 7 minutes respectively.

[2] All animals in this group received a daily dose of 2.5γ per gm. body weight of desoxycorticosterone acetate (Cortate, Schering) during 8 days, and were killed on the 9th day.

fractions of the total results obtained with the method applied in my experiments in the following order.

Spleen: 8–12% of the average AC values (summer and winter)
Kidney: 11–12% of the average AC values (summer and winter)
Liver: 15–23% of the average AC values (summer and winter)
Brain: 16–28% of the average AC values (summer and winter)

The kidneys and livers of gray rats contained considerably larger amounts of AC than those of white rats (both determined during the summer months). The specificity ratios were about the same in both races (table 2).

Effects of Injections of Adrenalin and Desoxycorticosterone and of Adrenalectomy

Intramuscular injection of adrenalin (2.5γ per gm. body weight) was followed within an average interval of 19 minutes by a moderate rise of the AC content of muscle, liver, kidney and brain, while the AC content of the spleen remained practically unchanged (table 3). In contrast to muscle and spleen in which the sp.r. remained unchanged, an increase of the sp.r., indicating a higher adrenalin concentration, occurred in liver, kidney and brain tissue. Analogous changes were observed in the kidneys and livers of grey rats within an average of 13 minutes (table 2).

TABLE 4

Adrenalectomy[1]

Rat No.	Date	Days of survival	Kidney		Liver		Brain		Spleen		Muscle	
			c.u.	sp.r. 1:	c.u.	sp.r. 1:	c.u.	sp.r. 1:	c.u.	sp.r. 1:	c.u.	sp.r. 1:
85	11/12	5	1109	1.06	1207	1.29	1287	0.89	609	1.34	1031	2.06
86	1/18	5	984	0.87	1243	1.16	1509	1.00	1554	1.06	722	1.22
87	1/24	6	1496	0.88	1148	0.85	2384	1.02	2459	0.93	1177	1.79
88	1/14	8	1676	1.25	1311	—	2016	0.95	1627	0.89	744	1.42
89	1/28	10	869	1.53	1545	1.09	1287	0.98	1408	1.09	592	1.65
90	1/29	11	1784	1.21	1652	0.97	1664	1.06	1436	0.82	835	1.35
91	11/13	12	1032	0.99	998	1.04	2108	1.03	1045	1.48	1109	1.19
92	1/14	16	946	1.59	1197	1.12	1107	1.00	1660	1.25	959	1.35
93	11/30	54	1696	1.16	1809	0.87	2065	1.03	1796	1.11	659	1.44
Average		14	1288	1.06	1345	1.05	1714	1.00	1510	1.11	870	1.50
Winter standard			1377	1.08	2005	1.05	2256	0.93	2855	1.00	1074	1.99
Deviation			−7%		−33%		−24%		−47%		−19%	

[1] All animals of this group died spontaneously. In no instance were remnants of suprarenal tissue found at autopsy. *Rats* 88 and 93 had been castrated one day before their death, *rat* 92 eight days before its death.

Desoxycorticosterone acetate[3] was administered intramuscularly to white rats daily during an 8-day period, a total dose of 20γ per gm. body weight. Kidneys and livers were examined. They showed a marked rise of the AC concentration (table 3) but no significant shift of the sp.r. toward the adrenalin side, in logical contrast to their behavior after injection of adrenalin.

[3] Cortate, Schering.

Adrenalectomy was performed in a series of white rats during the winter months (table 4) and the results obtained in these animals should be compared, therefore, with 'winter standard' figures. Animals which died within the first 48 hours after operation were discarded. Nine rats were examined following spontaneous death, the shortest duration of survival having been 5 days, the longest 54 days. There was a reduction of AC in all organs, however, only of a moderate degree, and only in a very few exceptional instances AC levels lower than in the lowest normal summer results were found. The average AC concentrations in the adrenalectomized rats were even slightly higher than in the normal summer rats with the exception of the spleen which showed both the highest AC level under normal conditions and the greatest AC loss after adrenalectomy. The sp.r. remained practically unaltered after adrenalectomy except in the muscle where it shifted somewhat toward the sterol side.

Effects of Testosterone, Castration, Insulin and Thyroxin

Administration of the male sex hormone (testosterone propionate,[4] 1 mg. per day for 8 days) did not alter the AC of kidney and liver and their sp.r. significantly (table 5).

TABLE 5

Injection of testosterone propionate[1]						Castration[2]					
Rat No.	Date	Kidney		Liver		Rat No.	Date	Kidney		Liver	
		c.u.	sp.r. 1:	c.u.	sp.r. 1:			c.u.	sp.r. 1:	c.u.	sp.r. 1:
94	2/18	1409	1.04	1787	1.00	100	2/14	1838	1.11	2470	1.00
95	2/18	1167	1.06	2055	0.93	101	2/14	1168	0.90	2064	0.98
96	2/19	904	1.04	2409	1.00	102	2/21	1010	1.26	1829	1.08
97	2/19	1350	1.00	2584	1.10	103	2/21	989	1.77	1992	1.42
98	2/20	1199	1.00	2134	0.96	104	2/22	1451	1.27	1358	0.96
99	2/20	1150	1.00	1980	1.00	105	2/22	1157	1.18	2207	0.98
Average		1196	1.02	2158	1.00	Average		1269	1.25	1987	1.07
Winter standard		1377	1.08	2005	1.05	Winter standard		1377	1.08	2005	1.05
Deviation		−13%		+8%		Deviation		−8%		−1%	

[1] All animals of this group received daily 1 mg. of testosterone propionate (Perandren, Ciba) for 8 days.

[2] *Rats* 100 and 101 were killed 10 days after castration, 102 and 103 after 14 days and 104 and 105 after 15 days.

Also castration (table 5) hardly changed the AC content of kidney and liver within an average of 13 days and the very slight alterations of the sp.r. seemed insignificant.

Insulin (table 6; 3 to 18 u) increased the AC content of liver, brain and muscle with a slight shift of the sp.r. toward adrenalin in all of these organs.

Thyroxin (table 6; 3 mg. distributed over 3 days) did not affect the AC content of liver and brain appreciably but it reduced the AC content of the muscles 23% and increased their relative adrenalin concentration.

[4] Perandren, Ciba.

TABLE 6

Injection of insulin[1]									Injection of thyroxin[2]							
Rat No.	Date	U	Liver		Brain		Muscle		Rat No.	Date	Liver		Brain		Muscle	
			C.U.	sp.r. 1:	C.U.	sp.r. 1:	C.U.	sp.r. 1:			C.U.	sp.r. 1:	C.U.	sp.r. 1:	C.U.	sp.r. 1:
106	8/27	6	2057	0.69	1743	0.97	1231	1.33	*116*	9/17	1094	1.14	1430	0.92	561	4.87
107	8/27	5	1706	1.17	2034	1.00	514	1.93	*117*	9/17	680	—	1208	0.89	167	1.00
108	8/28	8	2092	0.93	1915	0.94	686	1.22	*118*	9/18	1213	0.94	1145	0.93	892	3.70
109	8/28	12	2281	1.16	1633	1.03	867	1.69	*119*	9/18	1430	1.16	1229	0.95	421	1.09
110	8/30	8½	1328	1.06	1606	0.95	1028	1.71	*120*	9/19	1448	1.21	1423	0.96	609	2.66
111	8/30	6½	1411	1.23	1216	1.00	376	2.93	*121*	9/19	1113	1.17	1300	0.82	417	2.31
112	8/31	15	1556	0.68	1849	1.07	1032	3.52	*122*	9/20	1554	0.91	1724	0.90	901	2.39
113	8/31	18	2164	1.23	1348	1.07	701	3.44	*123*	9/20	1655	1.29	1263	0.85	402	0.82
114	9/1	4	1555	0.78	1853	—	639	2.70	*124*	9/21	1012	1.37	1438	0.96	451	3.76
115	9/1	3	2584	1.39	1366	1.02	1387	3.47	*125*	9/21	914	0.65	1308	1.00	525	4.00
Average		8½	1873	1.03	1656	1.00	846	2.39	Average		1211	1.09	1347	0.92	535	2.66
Summer standard			1335	0.96	1293	0.85	703	1.77	Summer standard		1335	0.96	1293	0.85	703	1.77
Deviation			+40%		+28%		+20%		Deviation		−9%		+4%		−23%	

[1] The animals of this group were killed at various intervals after the injection of insulin: *106*, *110* and *114* after 40–60 minutes; *107*, *108*, *111*, *115* after 80–120 minutes; *109*, *112*, *113* after 150–200 minutes. Seven of these rats were drowsy but none developed convulsions.

[2] All animals of this group received 1 mg. of thyroxin during 3 days and were killed on the 4th day.

TABLE 7

Exposure to cold[1]				Exposure to heat[2]				Enforced physical exercise[3]											
Rat No.	Date	Muscle		Rat No.	Date	Muscle		Rat No.	Date	min.	Muscle		Rat No.	Date	min.	Muscle		Rat No.	Date	min.	Muscle	
		c.u.	sp.r. 1:			c.u.	sp.r. 1:				c.u.	sp.r. 1:				c.u.	sp.r. 1:				c.u.	sp.r. 1:
126	9/22	866	0.71	136	9/25	405	3.36	146	9/11	6	491	4.10	156	9/7	45	330	0.81	166	9/10	260	69	0.90
127	9/22	475	1.77	137	9/25	367	—	147	9/13	8	760	1.60	157	9/9	50	813	1.47	167	9/13	300	885	3.37
128	9/22	454	3.30	138	10/29	1367	1.64	148	9/11	10	470	4.38	158	9/9	80	711	3.45	168	9/10	310	887	3.80
129	9/23	132	3.33	139	10/29	1101	1.58	149	10/15	10	407	2.65	159	9/9	120	451	0.69	169	9/13	320	412	2.33
130	9/23	563	5.33	140	10/29	1084	1.10	150	10/15	10	904	1.73	160	10/9	180	758	1.90	170	9/13	400	48	3.50
131	9/23	465	4.10	141	10/30	611	1.05	151	10/15	10	415	4.00	161	10/9	210	326	4.55	171	9/13	420	246	1.08
132	9/23	598	1.24	142	10/30	708	1.75	152	10/15	10	394	3.25	162	10/26	120	828	1.33	172	10/25	500	373	1.00
133	9/24	808	4.60	143	10/30	567	3.27	153	9/11	15	673	4.50	163	10/26	135	1406	2.45	173	10/25	520	569	1.93
134	9/24	614	3.33	144	10/31	555	1.00	154	9/9	20	955	3.86	164	10/26	150	497	0.77	174	10/25	540	332	3.00
135	9/24	236	2.00	145	10/31	541	1.10	155	9/7	15	604	3.69	165	10/26	160	836	1.17	175	10/25	560	316	1.11
Average		521	2.97	Average		731	1.56	Average		11	607	3.38	Average		125	696	1.86	Average		410	414	2.20
Summer standard		703	1.77	Summer standard		703	1.77	Summer standard			703	1.77	Summer standard			703	1.77	Summer standard			703	1.77
Deviation		−26%		Deviation		+4%		Deviation			−14%		Deviation			−1%		Deviation			−41%	

[1] All animals of this group were exposed for 10 minutes to water at 5°C. At the end of this time they were drowsy and stiff.
[2] All animals of this group were exposed for 10 minutes to water at 45°C. At the end of this time they were drowsy and weak.
[3] All animals of these three groups were forced to swim around continuously in a large basin filled with water of approximate body temperature for periods of time indicated in the table. The animals of the third group showed symptoms of fatigue.

Effects of Cold and Heat and of Physical Exercise

Exposure to cold (table 7; 10 minutes in water of 5°C.) had almost the same effect upon the muscles as thyroxin, both in regard to the absolute AC concentration and the sp.r.

A warm temperature (table 7; 10 minutes in water of 45°C.) on the other hand, caused no significant changes in the muscle.

Enforced physical exercise (table 7; swimming in a large basin with luke-warm water for periods from 6 minutes to 9 hours) caused a slight diminution of the AC concentration in the muscles at the beginning. After several hours, when the animals became visibly exhausted and their movements awkward, this diminution reached a marked degree. The sp.r. indicated a considerably increased adrenalin concentration, particularly during the first minutes of excitement and vigorous exercise. Several sp.r. quotients, exceeding 3.5, in this group are not necessarily incompatible with the presence of pure free adrenalin, as Shaw (2) found a quotient of 4.0 in some of his adrenalin tests.

DISCUSSION

The findings reported here indicate the presence of considerable amounts of AC compounds (suprarenal cortical sterols tied together with adrenalin) in the parenchymatous organs of the rat and to a lesser extent also in the muscles of the extremities where a comparatively large concentration of 'free' adrenalin was found instead. (Conditions in the heart muscle will be discussed separately.) The findings show clearly the absorption of both injected adrenalin and desoxycorticosterone by the tissues mentioned and a diminution of tissue AC after adrenalectomy.

The comparatively long survival of rats after adrenalectomy (4) seems to be partly due to the slowness of AC depletion. The presence of relatively high, even though diminished, AC concentrations in the tissues of adrenalectomized rats at the time of their spontaneous death suggests that some qualitative alterations of the molecular structure of the cortical sterols which are deposited in the tissues have occurred after adrenalectomy, thus interfering with their biological efficiency in a manner which is incompatible with the maintenance of life. The adrenalin concentration in the tissues can probably be maintained through local adrenal information (5) even after removal of both suprarenal glands.

Determinations of the blood AC level before and after adrenalectomy were made in a 39 lb. dog (fig. 1). It was 165 color U per cc. before removal of the left gland alone. During the following 10 days it rose to 270 C.U. Immediately after removal of the second gland there was a further slight rise, followed by a steady fall which was still continuing about 10 hours before death, when the last blood specimen was taken. At this time it was only slightly lower than the original normal level. The sp.r., determined once, 22 hours after the second operation, gave no evidence of 'free' adrenalin.

The absence of significant changes in the AC concentration of the organs after administration of testosterone propionate and after castration suggests that the sterols which participate in the physiological formation of AC compounds are exclusively of suprarenal cortical origin.

Insulin, which is known to stimulate suprarenal secretion (6, 7, 8), causes an accumulation of AC rich in adrenalin in the tissues.

Thyroxin exerts little effect except for a moderate diminution of muscular AC on the expense of cortical sterols while adrenalin is relatively increased.

Exposure to cold causes changes in the muscle, analogous to those brought about

by thyroxin, while warm temperatures are practically ineffective, as far as the muscles are concerned.

Prolonged enforced muscular exercise causes a slow diminution of the total muscular AC, while the relative adrenalin concentration is high. This behavior is also analogous to that elicited by cold and by thyroxin.

There are marked seasonal and racial peculiarities of the AC concentration in the organs in that it is considerably higher in all tissues during the winter months and in dark pigmented animals. The latter fact corresponds with findings (9, 10) concerning the larger size of the suprarenal glands in dark pigmented rats, pigeons and horses.

The spleen seems to possess a special functional role as a storage organ for AC

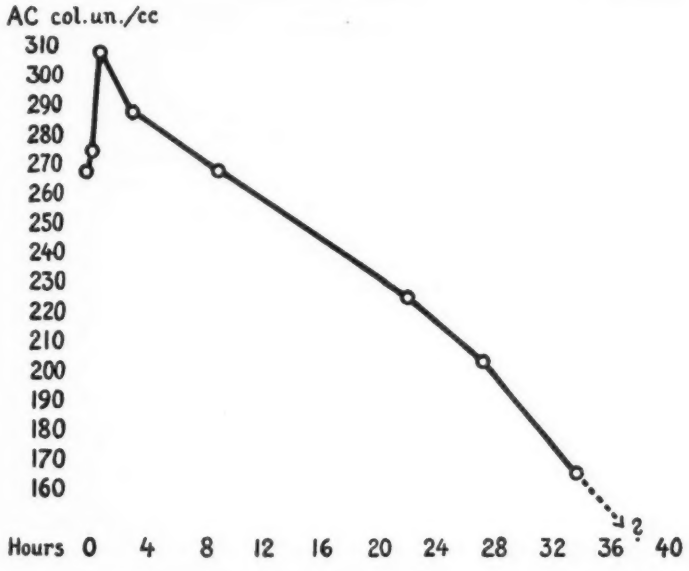

Fig. 1. Blood AC levels of a dog immediately before and following removal of the left suprarenal gland, the right one having been removed 10 days previously.

compounds. Its AC concentration is the highest of all organs, its AC losses after adrenalectomy are greater than those of any other organ and it is the only one whose AC concentration does not increase after injection of adrenalin. It seems possible that the AC stores of the spleen are drawn upon after adrenalectomy in order to counteract AC depletion in other tissues of the body.

Significant amounts of 'free' adrenalin, surpassing the quantities bound to sterols were found only in the muscle, except after adrenalin injection, when some free extra-adrenalin appeared also in the liver and in the kidneys. (Conditions in the heart muscle differed in various ways from those in other tissues.)

The apparent universal importance of the suprarenal substances, and in particular of the cortical sterols, for cellular oxydative processes, for carbohydrate, water and mineral metabolism (11, 12, 13), is emphasized by their presence in various kinds of tissue, their indispensability for the maintenance of life and the peculiarities of their distribution under various conditions.

SUMMARY

A method for the quantitative chemical assay of adreno-cortical (AC) hormones in tissues and of their qualitative composition is described.

Considerable amounts of AC were found in the tissues of muscle, kidney, brain, liver and spleen of the rat. (The findings in the heart will be published separately.)

Seasonal and racial variations and characteristic reactions to experimental conditions, such as administration of hormones (adrenalin, desoxycorticosterone, testosterone, insulin, thyroxin), adrenalectomy, castration, physical exercise, exposure to cold and warm temperatures, are discussed.

The spleen seems to possess a special function as a storage and supply organ for AC reserves.

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ASSAY OF INSULIN ON HYPOPHYSECTOMIZED, ADRENO-DEMEDULLATED, AND HYPOPHYSECTOMIZED-ADRENO-DEMEDULLATED RATS[1]

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ADRENALECTOMIZED RATS and mice have been used by Hemmingsen, Nielsen and Nielsen (1) for the assay of small quantities of insulin. The criterion chosen by these authors, the occurrence of hypoglycemic convulsions, did not permit them to assay insulin very accurately because of marked individual differences in the convulsive threshold of various animals. By using the hypoglycemic effect as an index, minute quantities of insulin could be detected in the blood of adrenalectomized rats which had been subjected to anoxia or metrazol (2).

In the present investigation hypophysectomized, adreno-demedullated, and hypophysectomized-adreno-demedullated rats have been used systematically for the assay of insulin both by itself and in the presence of blood.

METHOD

Rats weighing from 100–130 gm. were used. In one group the adrenals were demedullated and the rats were allowed to drink 2% NaCl. Another group was hypophysectomized by the pharyngeal approach and in the third group the adrenal medullae were removed 8 days after hypophysectomy. This group was also allowed to drink 2% NaCl and 2% glucose and was used for the experiment 3 to 4 days after the second operation. The other groups were used one or several weeks after the operation. The time interval had apparently no effect on their reactivity to insulin. The hypophysectomized and the adreno-demedullated rats were fasted 18 hours before the insulin assay was undertaken, whereas in the third group (hypophysectomized-adreno-demedullated rats) the fast was only 6 hours.

Insulin Lilly[2] was injected intraperitoneally. If blood was used it was injected with insulin within 5 minutes after it had been withdrawn. Sixty minutes after the injection of insulin a blood sample was taken from the tail or the heart and the sugar determined by the Somogyi modification of the Shaffer-Hartman method.

RESULTS

Figure 1 shows the effect of various quantities of insulin on the blood sugar of hypophysectomized and of adreno-demedullated rats. The blood sugar falls fairly linearly in response to increasing concentrations of insulin. Convulsions or paresis occurred with 0.02 U of insulin per 100 gm. of body weight in adreno-demedullated and with 0.025 to 0.03 U of insulin per 100 gm. in the hypophysectomized rat. The

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[2] Kindly supplied by the Eli Lilly Company.

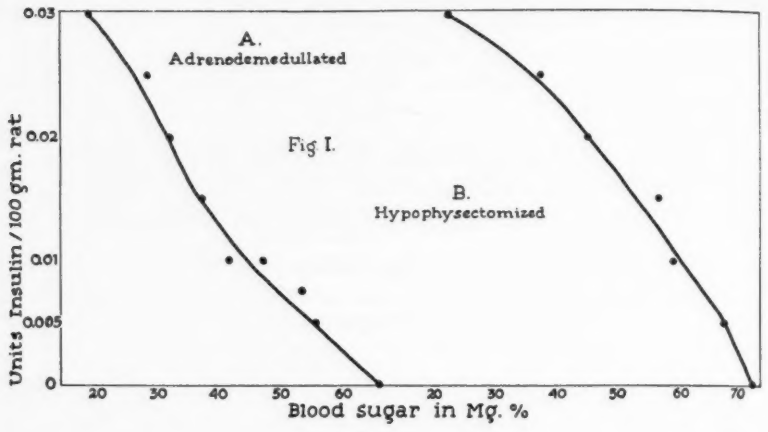

FIG. 1

smallest effect determinable was about 0.005 U per 100 gm. of body weight in both groups but the fall in blood sugar was slightly greater in the adreno-demedullated rat.

The third series was performed on hypophysectomized-adreno-demedullated rats. Figure 2 shows that the combination of the two operations resulted in a remarkable

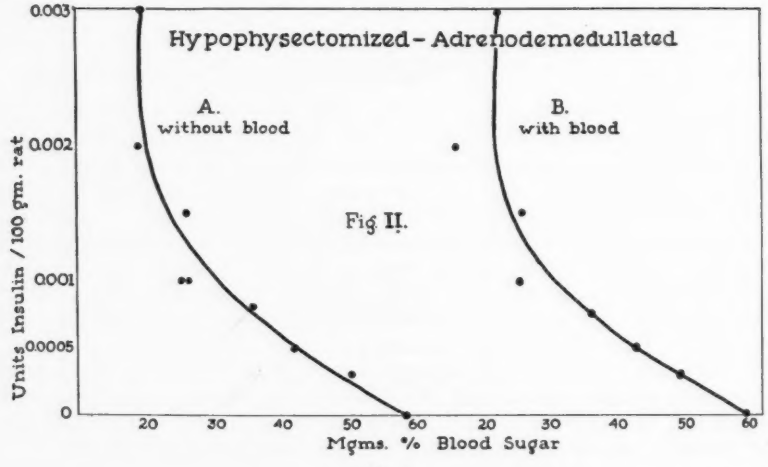

FIG. 2

increase in the sensitivity of these animals to insulin; 0.0003 U insulin per 100 gm. of body weight led to a distinct fall in blood sugar. The curve shows an approximately linear relation between the blood sugar and the insulin concentration up to 0.001 U insulin per 100 gm. of body weight which represents the convulsive threshold. The curve then turns sharply since even doubling or trebling the insulin does not greatly alter the blood sugar reaction. The minimal value of about 19 mg.% is obtained with both 0.002 and 0.003 U insulin per 100 gm. rat.

If various quantities of insulin are added to 1 cc. of normal human blood they produce approximately the same quantitative hypoglycemic effects as in the absence of

blood. Obviously, blood does not appreciably alter the rate of absorption of insulin from the peritoneal cavity of the hypophysectomized-adreno-demedullated rat (fig. 2).

Seven control experiments in which the influence of 1 cc. of normal human blood (obtained 2½ hours after luncheon) was tested on the hypophysectomized-adreno-demedullated rat showed a slight but consistent fall in blood sugar (table 1). It is

TABLE 1. INFLUENCE OF THE INTRAPERITONEAL INJECTION OF 1 CC. OF HUMAN BLOOD PER 100 GM. OF BODY WEIGHT ON THE BLOOD SUGAR OF THE NORMAL AND OF THE HYPOPHYSECTOMIZED-ADRENO-DEMEDULLATED RAT

	Normal Rat	
	Blood sugar mg. %	
No.	Before Injection of blood	60 Min. after
1	77.4	79.5
2	75.2	78.5
3	78.5	78.5
4	76.3	74.1
Mean	76.8	77.4

	Hypophysectomized-Adreno-Demedullated Rat	
	Blood sugar mg. %	
No.	Before Injection of blood	60 Min. after
1	56.9	52.6
2	56.9	53.7
3	58.0	49.4
4	56.9	48.3
5	56.9	51.6
6	59.1	53.7
7	56.9	50.5
Mean	57.4	51.4
St. Dev.		1.94

assumed that this effect is due to minute amounts of insulin in the normal blood since blood has no effect on the blood sugar of normal rats. Furthermore it was found that the effect on the blood sugar of hypophysectomized-adreno-demedullated rats is the same whether the vagi are intact or have been sectioned below the diaphragm. Apparently the blood does not act appreciably on the centers of the vago-insulin system since then the hypoglycemic effect would depend on the integrity of the right vagus. Since it has been shown that typhoid vaccine (3, 4) acts on the center of the vago-insulin system it may be inferred that various foreign proteins differ considerably in their affinity to the autonomic centers. On the basis of this assay the insulin content of the normal human blood seems to be about 0.0002 U of insulin per cc. This value agrees with the observation that the injection of 4 cc. of human blood into hypophysectomized-adrenalectomized rats causes a fall in blood sugar to about 43 mg. %, indicating about 3 times the amount of insulin found in 1 cc. of blood.

In view of this exceedingly small amount of insulin in normal human blood it is not surprising that other attempts have failed to determine it. Brugsch and Horsters (5) who used the normal mouse as a test animal were able to conclude only that the insulin content of human blood is less than 0.05 to 0.06 U per cc.

It is worthy of note that the combination of demedullation of the adrenals with hypophysectomy increases the sensitivity of the rat to insulin much more than would be expected if the two procedures should act independently of each other. The nature of this interaction is still unknown.

The mouse is considerably more sensitive to insulin than the rat (1). In our earlier work (2) we found that the adrenalectomized mouse allows detection of about 0.0015 U of insulin, whereas in the adrenalectomized rat quantities smaller than 0.005 U

could not be detected. It seems not unlikely that if the procedures used in this investigation on the rat were applied to the mouse it would further improve the assay of insulin.

Although the results obtained with this method will be reported elsewhere, it may be mentioned that psychotic patients in periods of excitement showed regularly an increased insulin concentration in the blood in spite of the unchanged blood sugar level. Furthermore, in two cases of the hyperinsulinism an increased amount of insulin was found in the blood by means of the assay in the rat.

SUMMARY

Sensitivity to insulin as measured by the fall in blood sugar is slightly greater in adreno-demedullated than in the hypophysectomized rat. Two hundredths U insulin per 100 gm. of body weight causes convulsions or coma in the adreno-demedullated rat and 0.025–0.03 U per 100 gm. of body weight have the same effect on the hypophysectomized rat. By combining these procedures in the same animal the insulin sensitivity is greatly increased so that even as little as 0.001 U insulin per 100 gm. of body weight causes hypoglycemic convulsions or coma. The insulin content of normal human blood is approximately 0.0002 U per cc. If known quantities of insulin are added to human blood they produce quantitatively similar effects on the hypophysectomized-adreno-demedullated rat as in the absence of blood. The method allows determination of alterations in the insulin concentration of the human blood.

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THE INFLUENCE OF FEVER ON THE VAGO-INSULIN AND SYMPATHETICO-ADRENAL SYSTEMS[1]

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THERE is evidence in the literature that fever produced by the injection of bacterial toxins acts on both the vago-insulin and sympathetico-adrenal systems. Cannon and Pereira (1) showed that typhoid vaccine causes fever and an increased adrenalin secretion. Geiger (2) observed that colitoxin produced fever and hyperglycemia, but he found also evidence of an excitation of the vago-insulin system since during the time in which the temperature returned to normal a hypoglycemia occurred in rabbits. This effect was absent in rabbits in which the vagi had been cut previously, but not in dogs under similar conditions (2). La Barre (3), however, showed in dogs that injection of polyvalent vaccine produced fever and increased insulin secretion mediated by the vagus. He assayed the insulin concentration of the blood by the blood sugar reaction in cross-circulation experiments. Ranson (4) observed in cats, in which fever was produced by typhoid vaccine, that the rise in temperature is not smooth but shows marked oscillations indicating temporary phases with predominance of the parasympathetic control (heat loss), although the general tendency is a rise in temperature with increased heat production (sympathetico-adrenal effects).

In the present study an attempt was made to investigate the influence of typhoid-paratyphoid vaccine on the vago-insulin and sympathetico-adrenal systems by the study of the blood sugar and to correlate these effects with the temperature of the body. Typhoid-paratyphoid vaccine[2] was injected intraperitoneally (0.7 cc./kg.). As in several previous papers of Gellhorn and collaborators (5, 6), the effect was studied on the blood sugar in normal, adreno-demedullated, adreno-demedullated-vagotomized, and vagotomized rats. The temperature was measured rectally.

RESULTS

Effect on blood sugar. Normal rats reacted to the typhoid vaccine with a rise in blood sugar which reached its maximal effect after 2 hours and gradually returned to the control level after 5 hours. Adreno-demedullated rats reacted with a steadily increasing hypoglycemia, the maximal effect having been obtained at the end of the experimental period. Adreno-demedullated-vagotomized rats showed no significant change in the blood sugar level. These results indicate that both sympathetico-adrenal and vago-insulin systems are excited and that the excitation of the former predominates in the normal animal. The elimination of the adrenal medulla creates conditions in which the typhoid vaccine causes a hypoglycemia due to the excitation of the vago-insulin system. If this interpretation is correct then the elimination of the vago-insulin system by subdiaphragmatic vagotomy should lead to a more marked hyperglycemia than is observed in control animals. The experiments on vagotomized rats showed a marked prolongation of the hyperglycemic reaction; even after 6 hours the blood sugar had not returned to the control level. The maximal blood sugar rise occurred after 2 hours and was slightly greater than that observed in the control group.

Effect on body temperature. There was a remarkable parallelism between the reaction of the blood sugar and the temperature of the body. The normal and the vagot-

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[2] Abbott Laboratories.

THE INFLUENCE OF TYPHOID-PARATYPHOID VACCINE[1] ON THE BLOOD SUGAR

Rat No.	Control		1 Hour		2 Hours		3 Hours		4 Hours		5 Hours		6 Hours	
	Blood sugar	Body temp.	Blood sugar	Body temp.	Blood sugar	Body temp.	Blood sugar	Body temp.	Blood sugar	Body temp.	Blood sugar	Body temp.	Blood sugar	Body temp.
						A. Normal Rats								
	mg. %	°F.	mg. %	°F.	mg. %	°F.	mg. %	°F.	mg. %	°F.	mg. %	°F.	mg. %	°F.
1	74.1	101.2	79.5	101.8	105.3	103.5	102.1	103.0	94.6	101.2	73.1	99.8	68.8	99.6
2	77.4	102.1	83.8	102.6	96.8	103.8	94.6	103.6	83.8	102.0	73.1	100.8	64.5	101.0
3	72.0	100.3	81.1	100.6	98.9	102.0	83.8	100.4	75.2	99.8	66.6	96.1	66.6	98.0
4	74.1	98.8	80.6	99.8	107.5	102.1	105.3	101.5	83.8	98.6	74.1	97.4	68.8	97.0
5	70.9	100.0	75.2	101.7	96.8	102.3	102.1	103.4	86.0	102.0	73.1	100.4	62.3	98.8
6	75.2	100.6	83.8	101.4	101.0	102.8	91.3	100.4	74.1	98.3	67.7	97.6	70.9	98.8
Mean	72.9	100.5	80.7	101.3	101.1	102.8	96.5	102.1	82.9	98.8	71.3	98.7	67.0	98.9
Stand dev.	2.4		2.9		4.1		7.4		6.9		3.0		2.8	
P			<0.01		<0.01		<0.01		<0.01		0.33		<0.01	
						B. Adrenalectomized Rats								
1	68.8	98.8	64.5	98.0	56.9	97.6	50.5	95.0	48.3	93.2	45.1	92.2	40.8	92.2
2	65.5	99.2	59.1	98.6	51.6	97.2	45.1	95.3	43.0	93.8	40.8	92.4	37.6	92.3
3	63.4	98.6	59.1	98.0	46.2	97.8	41.9	96.0	39.7	92.5	36.5			
4	64.5	99.6	58.0	99.0	52.6	96.9	48.3	94.4	45.1	92.4	40.8	92.1	37.6	92.1
5	64.5	100.7	58.0	98.8	51.6	95.8	49.4	94.2	43.0	93.8	40.8	93.0	36.5	92.4
6	60.2	99.6	58.0	98.5	53.7	96.4	51.6	95.6	48.3	94.8	43.0	93.5	40.8	92.9
Mean	64.5	99.4	59.5	98.5	52.1	96.9	47.9	95.1	44.6	93.6	41.2	92.6	38.7	92.4
Stand dev.	2.5		1.3		3.2		3.1		3.1		2.6		1.8	
P			<0.01		<0.01		<0.01		<0.01		<0.01		<0.01	
						C. Adrenalectomized-Vagotomized Rats								
1	68.8	98.1	70.9	97.9	72.0	97.1	69.8	96.5	67.7	96.2	69.8	95.8	68.8	95.5
2	64.5	99.0	70.9	98.7	73.1	98.0	70.9	97.7	68.8	97.4	68.8	96.5	68.8	95.9
3	65.5	98.0	73.1	98.6	78.5	99.0	75.2	98.3	74.1	96.5	74.1	96.3	73.1	95.6
4	70.9	97.8	70.9	97.1	73.1	96.8	68.8	96.5	70.9	96.0	72.0	95.7	72.0	95.3
5	64.5	97.6	69.8	96.4	69.8	96.0	73.1	96.5	70.9	95.1	66.6	94.7	66.6	94.5
6	66.6	100.7	64.5	99.8	66.6	99.7	68.8	98.0	72.0	96.8	70.9	96.4	68.8	96.0
Mean	66.8	98.5	70.0	98.8	72.2	97.8	71.1	97.3	70.7	96.7	70.4	95.9	69.7	95.5
Stand dev.	2.3		2.7		3.6		2.3		2.1		2.4		2.2	
P			0.052		0.013		0.010		0.013		0.027		0.075	
						D. Vagotomized Rats								
1	68.8	99.8	86.0	100.6	107.5	101.1	86.0	102.2	84.9	103.0	79.5	102.6	81.1	103.1
2	72.0	97.7	90.3	101.9	110.9	101.7	99.9	103.7	91.3	101.8	84.9	101.9	82.7	100.7
3	73.1	100.0	80.0	101.4	99.9	101.8	89.2	101.9	83.8	102.6	81.1	102.6	77.4	102.8
4	73.1	99.0	88.2	100.7	106.4	101.6	102.1	101.7	90.3	102.7	84.9	101.8	80.6	101.6
5	77.4	98.7	89.2	101.9	102.1	102.2	110.0	102.7	97.8	101.5	90.3	101.3	84.9	101.1
6	72.0	99.7	86.0	102.7	104.2	103.1	104.2	102.8	87.0	103.1	84.9	103.1	78.5	102.3
Mean	72.7	99.2	86.6	101.5	105.2	101.9	98.6	102.2	89.2	102.5	84.3	102.2	80.9	101.9
Stand. dev.	2.9		3.4		3.6		8.4		4.7		3.4		2.5	
P			<0.01		<0.01		<0.01		<0.01		<0.01		<0.01	

[1] Paratyphoid vaccine (Abbott) was injected subcutaneously (.7 cc./kg.). It contains typhoid bacilli 1,000,000,000, paratyphoid A 750,000,000 and paratyphoid B 750,000,000/cc.

omized rats showed both hyperglycemia and hyperthermia. Just as the hyperglycemia was more prolonged in the vagotomized animals than in the controls because of the exclusion of the counteracting vago-insulin system in the former, so was hyperthermia more marked in the vagotomized than in the unoperated rats. Whereas in the control group the normal temperature was reached after 4 hours, the hyperthermia still existed after the end of 6 hours in the vagotomized rats. The remarkable parallelism between the hyperglycemia and the hyperthermia in these two groups suggests that just as insulin mobilized through vagal impulses counteracts to some extent the effect of sympathetico-adrenal excitation on the blood sugar, it may also diminish the temperature raising action of adrenalin. This interpretation is supported by a comparison of the reactions of adreno-demedullated and adreno-demedullated-vagotomized animals. The latter group showed a fall in body temperature gradually progressing to a maximum of 3° F. in 6 hours. Since the absence of changes in the blood sugar of this group testifies to the completeness of the elimination of the nervous regulation of insulin and adrenalin secretion in these animals, the fall in body temperature must be due to some peripheral effect of the vaccine. In the adreno-demedullated rats, however, in which the vagi were intact, a much more marked hyperthermia occurred progressing to a fall of 7° F. in 6 hours. This indicates that insulin liberated by vagal impulses caused a marked lowering in the temperature of the body.

The experiments prove that the stimulation of the autonomic centers is not due to the fever produced by the typhoid vaccine but that fever results from the stimulation of the sympathetico-adrenal system by typhoid vaccine in the normal and vagotomized animal. Furthermore, the typhoid vaccine acts also on the center of the vago-insulin system and causes both hypoglycemia and hypothermia in the adreno-demedullated animal. The greater rise in blood sugar and the prolongation of the rise in temperature in vagotomized animals as compared to unoperated controls clearly indicate that the vago-insulin system is excited during the fever in the normal animal and not only during the fall in the temperature of the body following fever as Geiger emphasized. Our observations are related to Ranson's (4) findings that at least in some of his cats with hypothalamic lesions, typhoid vaccine produced a fall in temperature instead of a rise. Unfortunately he did not study the blood sugar reaction to typhoid vaccine in these animals.

The results of our experiments are interesting in view of the fact that former investigations of Gellhorn and Feldman (6) have shown that cold and heat although acting on the sympathetico-adrenal as well as on the vago-insulin system excite these systems to a different extent in the two conditions, cold acting predominantly on the former whereas heat excites the vago-insulin system more than the sympathetico-adrenal system. The specificity of this adjustment reaction which is obviously adequate to maintain or restore homeostasis is no longer present under the pathological conditions of fever induced by typhoid-paratyphoid vaccine. It should, however, be mentioned that in investigations with various drugs such a bulbocapnine and cocaine no direct relationship could be observed between the stimulation of the autonomic centers and the temperature of the body. The observation of Gremels (7) that vagal stimulation in the spinal cat causes a fall in oxygen consumption and a decrease in the blood sugar level is probably an expression of the effect of vagal excitation on the liberation of insulin and on heat production.

SUMMARY

Typhoid-paratyphoid vaccine causes a hyperglycemia in the normal, a hypoglycemia in the adreno-demedullated, and no change in blood sugar in the adreno-demedullated-vagotomized rat. The experiments indicate that typhoid-paratyphoid vaccine causes a discharge over both sympathetico-adrenal and vago-insulin systems, the effect on the former predominating. This interpretation is supported by the fact that hyperglycemia is more marked and prolonged in the vagotomized rat when compared with the normal animal.

Similar relations appear in the study of the body temperature of these animals. Whereas normal and vagotomized rats react with hyperthermia, the adreno-demedullated rats show a hypothermia in response to typhoid-paratyphoid vaccine. The experiments prove that the excitation of the autonomic centers is not the result of fever but, on the contrary, variations in body temperature result from the injection of typhoid-paratyphoid vaccine by means of its effects on the vago-insulin and sympathetico-adrenal systems.

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EFFECTS OF DESOXYCORTICOSTERONE ACETATE IN THE ALBINO RAT[1]

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IT IS ESTABLISHED that some patients with Addison's disease given large doses of desoxycorticosterone acetate develop cardiac dilatation and signs of circulatory failure (1). Similar doses in normal dogs cause marked polydipsia and polyuria and result in periodic muscular weakness associated with very low serum potassium levels (2). The present study is concerned with the effects of prolonged administration of this drug in albino rats. In a preliminary experiment the treated rats failed to show effects similar to those seen in the human patient or in the dogs but changes in the weight of the testes and pituitary glands directed attention to the endocrine organs in subsequent groups.

EXPERIMENTS

Group I. Due to the striking effect of the drug on serum potassium levels of human patients and dogs, the first group of rats was maintained on a low K diet (3) throughout the period of observation. A total of 11 rats of both sexes, weighing 104 to 180 gm. at the start of treatment, each received 5 mg. desoxycorticosterone acetate in 1 cc. peanut oil daily for 8 weeks. The 16 controls on the same diet selected for similar size and same sex, weighed 108–160 gm. Some of these received 1 cc. peanut oil daily for the same period and the others were not injected.

Group II. Since the symptoms in the dogs had been accentuated by a high NaCl intake, a small group of rats was maintained on our stock diet plus 4% NaCl while being treated for a period of 8 weeks. In this group there were 4 males, weighing 320 to 370 gm., each of which received 5 mg. desoxycorticosterone acetate in 1 cc. peanut oil daily. Two control males, 332 and 350 gm. on the same diet, received 1 cc. of oil alone daily.

Group III. Because the endocrine effects of long maintenance on the abnormal diets were not known, a third group of animals was given a normal (modified McCollum) diet[2] during the course of treatment. The treated animals of this group consisted of 12 males and females weighing 94 to 112 gm. at the start of treatment. In order to avoid large subcutaneous accumulations of oil the 5 mg. daily dose of desoxycorticosterone acetate was suspended in only 0.2 cc. of oil. The period of treatment was shortened to 4 weeks. There were 12 well matched controls of the same sex, on the same diet, weighing 94 to 118 gm., each of which received 0.2 cc. peanut oil daily.

Body weights were recorded at intervals. Water consumption was measured by

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[1] The desoxycorticosterone acetate, Doca, was supplied by the Roche-Organon Laboratories, Inc., Nutley, N. J.

[2] Ground whole wheat 65.0%, casein (comm.) 15.0, whole milk powder 10.0, Wesson oil 5.5, cod-liver oil 2.0, $CaCo_3$ 1.5, NaCl 1.0.

groups during chosen periods, both with and without the addition of 0.9% NaCl. The animals were autopsied the day following the last injection. The thymuses, endocrine, and accessory sex organs were dissected out and weighed and histological sections were made of all these as well as heart, liver, kidney, spleen, skin and skeletal muscles. Some of the adrenals were fixed in Maximow's fluid and stained with osmic acid according to the technic of Flexner and Grollman (4). The posterior lobes of some of the pituitaries were also fixed in Maximow's fluid and impregnated with osmic acid to stain the granules in the parenchymatous cells (5). The anterior lobes of the pituitaries of certain of the animals were stained by the Severinghaus technic (6) after Zenker or Maximow fixation. Other sections were stained with hematoxylin and eosin after Zenker or Bouin fixation. The parathyroids of Group III were serially sectioned at 10 micra and the volumes determined by camera lucida drawings and planimeter measurements (7).

RESULTS

With the exception of one treated female of Group III none of the animals showed signs of illness. This animal sickened on the 5th day of treatment and was killed and autopsied 3 days later. No anatomical lesions were found. This animal was omitted from the tables. None of the animals showed signs of muscular weakness. Growth was not significantly affected by the treatment.

The water consumption of the treated animals on the normal diet (Group III) was approximately 50% greater than that of their controls over a 5-day period. In a desoxycorticosterone acetate treated group of animals on the high Na diet (Group II) the addition of 0.9% NaCl to the drinking water raised the daily water consumption to 300 cc. per animal. Their controls drank 150 cc. per animal under the same conditions.

Hearts. At autopsy the hearts of the animals in Groups I and II were weighed. They were variable, but did not differ significantly between treated and control groups. Sections of the hearts of the animals of all groups were essentially normal. A few of the animals on the low K diet had an occasional tiny myocardial scar, composed of fibroblasts and few, if any, inflammatory cells. These were found in four of the treated and two of the control animals. There were no fresh necroses.

The endocrine and accessory sex organ weights of Group III are recorded in the table. Similar results were obtained in Groups I and II but the wide variation in the ages of these animals prevents direct comparison.

Adrenals. In all groups the effect of the treatment on the adrenal glands was a marked reduction in their size. This effect was greatest in Groups I and II which were treated for a longer period of time. There was a symmetrical reduction in the width of the cortex due to a visible decrease in cellular volume (fig. 1). In some of the glands there were small amounts of hemosiderin pigment in phagocytes particularly in the inner cortical layer. The medulla was normal. There was only one constant and characteristic change in the distribution of osmic reducing substances in the treated animals. This was a nearly complete disappearance of the heavy osmiophilic deposit in the peripheral zone of the gland (fig. 2).

Thymus. The average thymus weights were always a little larger in the treated animals. The high variability of the thymus weights in all groups makes the difference of questionable statistical validity but the same difference was observed in each group. It was of the greatest magnitude in the oldest animals (not in the table) in whom the ratio of thymus to body weight was normally decreasing. There were no appreciable histological differences.

The *thyroids* were unaffected in weight or histological structure. The *parathyroid*

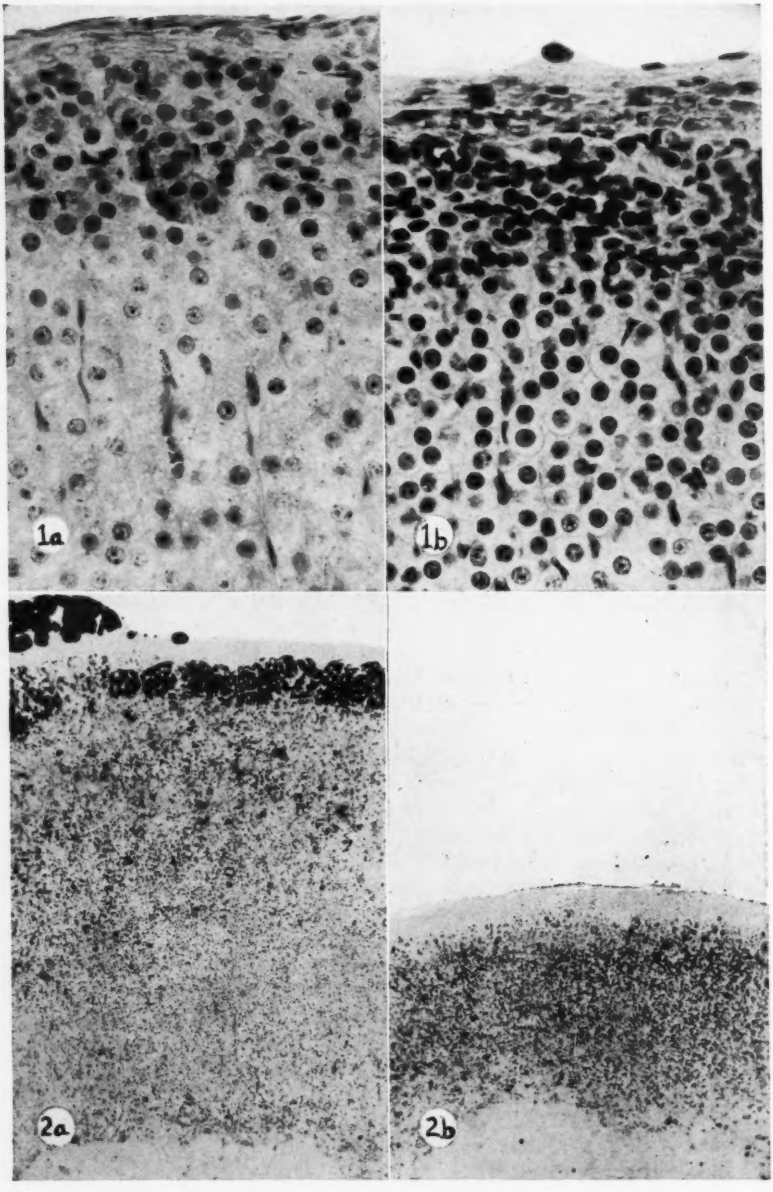

Fig. 1. Adrenal cortex of control (*a*) and treated (*b*) animals from group I. Note smaller size of cortical cells, particularly of subcapsular zone, in b. Hematoxylin and eosin ×435. Fig. 2. Entire thickness of adrenal cortex from control (*a*) and treated (*b*) animals of group I. Note narrow cortex and disappearance of heavy subcapsular osmiophilic zone in b. Maximow fixation. 2% osmic acid impregnation. Sections cut at 7 micra. ×104.

volume was not significantly altered. The *pancreatic islets* were normal in appearance. No special granule stains were employed on the pancreas.

Testes. The testes were not affected in size or histological structure except in a small preliminary group of animals which deserves special mention. This group consisted of 3 animals (weighing 155–180 gm.) treated for 8 weeks while on the low K diet (part of Group I). Before they were killed it was noted that the testes of all of them were very much smaller than those of the controls. Two of the animals were then killed. Their testes and pituitary glands were only about half the weight of the control organs. Histologically the testes resembled those of hypophysectomized animals (8), having no spermatogenic elements beyond the stage of spermatogonia and only small undifferentiated-looking interstitial cells. The third animal was taken off treatment and an immediate attempt was made to breed him to normal females. It was not until 6 weeks later that this animal finally proved to be fertile and by this time his testes had grown to normal size and at autopsy were found to be normal histologically.

Prostates and seminal vesicles. It was this experience which prompted the investigation of endocrine and accessory sex organ weights in subsequent groups. There was a consistent small decrement in the weights of prostate glands and seminal vesicles of all the treated males investigated. This was not accompanied by any clear cytological evidence of diminished secretory activity. The epithelial cells of the ventral prostatic lobes invariably contained the secretory clear zones.

Ovaries and uteri. Although the ovarian and uterine weights were slightly smaller in the treated females than in their controls, the ovaries of both alike were studded with corpora lutea and showed no appreciable histological differences. The histological appearance of uteri and vaginae was variable, evidently dependent upon differences in the phase of the estrous cycle.

Pituitary glands. The marked reduction in the weight of the pituitary glands observed in the preliminary treated group with small testes was never reproduced. The table shows a tendency to reduced pituitary weight in the treated females but

THE EFFECT OF 5 MG. DESOXYCORTICOSTERONE ACETATE DAILY FOR 28 DAYS ON THE SIZE OF THE ENDOCRINE AND ACCESSORY SEX ORGANS OF THE RAT

	Final body weight	Hypophysis	Adrenals	Thyroid	Thymus	Testes	Prostate	Seminal vesicles	Parathyroids
	gm.	mg.	mg.	mg.	mg.	gm.	mg.	mg.	mm.³
				A. Males					
Treated (6)	229 (210–240)	8.4 (7.8–9.0)	21.2 (16.8–28.2)	18.9 (15.2–21.4)	580 (437–759)	2.57 (2.42–2.68)	335 (256–400)	344 (215–476)	0.2034 (0.1715–0.2410)
Control (6)	243 (236–250)	8.9 (8.2–9.3)	33.6 (29.0–39.5)	18.9 (17.0–23.1)	528 (429–653)	2.62 (2.31–2.85)	393 (333–455)	475 (356–593)	0.1810 (0.1710–0.1955)
t[1]		1.85	4.67	—	0.95	0.58	2.00	2.50	1.90
				B. Females					
						Ovaries	Uterus		
						mg.	mg.		
Treated (5)	174 (162–182)	9.6 (7.9–11.9)	33.3 (26.0–42.8)	15.6 (11.8–18.3)	478 (398–546)	51.1 (45.0–56.0)	249 (185–315)		0.1862 (0.1530–0.2250)
Control (6)	174 (156–190)	11.8 (9.1–14.9)	47.6 (40.5–52.5)	16.5 (12.6–21.5)	443 (365–532)	58.1 (52.5–69.0)	280 (2.7–441)		0.1773 (0.1472–0.2510)
t[1]		2.16	4.18	0.045	0.94	2.26	0.72		0.438

[1] R. A. Fisher and F. Yates "Statistical tables for biological, agricultural, and medical research," Oliver & Boyd, London 1938.

this is of questionable statistical significance. Differential counts of the anterior lobe cells were not made but the granule stains failed to show any obvious change as a result of the treatment. The size and abundance of osmiophilic granules in the parenchymatous cells of the posterior lobes varied considerably between individuals but were not appreciably different in the treated as compared to the control group.

Sections of several different *muscles* were examined and showed no alteration except locally in the proximity of the subcutaneous pockets of oil. These were marked by a striking accumulation of eosinophil leucocytes and fibroblastic proliferation. The other organs contained no lesions except frequent infiltrations of eosinophils, especially in the spleens, and these were alike in all the oil injected animals.

DISCUSSION

The most conspicuous effect produced by desoxycorticosterone acetate in these experiments was a decrease in adrenal size. This is a property of many potent adrenal cortical preparations (9, 10) although it does not appear to be strictly proportional to their life maintaining power (10). This effect has been previously reported by Selye (11) following large doses of desoxycorticosterone acetate although shorter treatment with smaller doses had earlier led Wells and Kendall (10) to deny that this was a property of this drug. The cellular changes in the small glands are essentially the same as those reported following treatment with adrenal cortical extracts (4, 9).

Thymic atrophy has been repeatedly observed (10, 12) following treatment with adrenal cortical preparations. However, this does not appear to be intimately related to the life maintaining power of these preparations (10). We therefore hesitate to cite its failure to occur in our animals as evidence of an important qualitative difference of desoxycorticosterone acetate from the other preparations.

The occurrence of marked testicular and pituitary atrophy in our preliminary group of animals was not subsequently reproduced. It seems probable that those animals represented an unusually susceptible group in our colony since Selye (13) has recently reported marked testicular atrophy with twice the dose of desoxycorticosterone acetate that we employed, or with even smaller doses provided the treatment was begun in day-old animals. The tendency to a decrease in pituitary weight, in our treated females particularly, must be confirmed by additional numbers. It might be anticipated that the testicular and pituitary effects are related but at present there is insufficient data to prove this. It was hoped that the weights of ovaries and accessory sex organs might throw additional light on the state of the pituitary-gonadal function. Although the weights of the ovaries and of the prostates and seminal vesicles are lower in the treated animals there is no histological evidence that the gonadal function was diminished in either sex, excluding the preliminary group.

The functional changes in the muscular and circulatory systems previously observed in human patients and in dogs were not manifest in the rats in these experiments. Increases in water consumption of a moderate degree were regularly produced in the treated rats. These are reminiscent of the syndrome of polydipsia and polyuria observed in desoxycorticosterone acetate treated dogs (2) but the magnitude of the effect was so much less as to discourage the use of the rat in the further study of this phenomenon.

SUMMARY

Prolonged administration of desoxycorticosterone acetate in large doses to rats failed to produce the evidences of toxicity previously observed in patients with Addison's disease and in normal dogs. The only striking effect of the treatment was a diminution in size of the adrenals due to atrophy of the cortical cells and accompanied

by a diminution of osmic reducing material in the peripheral zone. There was no thymic atrophy. No consistent change was observed in the other endocrine glands with the exception of a small diminution in pituitary weight which is of questionable statistical significance. One group of three animals had a marked reduction in both pituitary and testicular weight and histological evidence of testicular immaturity but that result was not reproducible with the dosage of the drug employed. Sections of the other viscera and the muscles failed to disclose any significant lesions.

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THE CORRELATION BETWEEN SEX AND CHEMICAL COMPOSITION OF THE CENTRAL NERVOUS SYSTEM OF WHITE RATS

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THE STUDY of the influence of the sex hormones has mostly been confined to morphology and physiology. Very little is known about their regulation of the chemical composition of the body, and when it comes to the study of their correlation with the central nervous system a review of the literature reveals the lack of any systemic investigation. There is the older work of Donaldson (1) and Hatai (2), which was concerned with the weight of the brain and its water content. The figures for the latter are given as 78.2 and 78.6% respectively for the normal male and the castrated rat (2) as compared with 76.5 and 76.4% respectively of the present paper. Such a marked difference is not surprising if one reads that their method of dehydration consisted in "drying the brain in a closed water bath at 85 to 95°C. for an average of 7 days." This compares with the present method of acetone dehydration for half a day at room temperature and drying at temperatures not exceeding 50°C.

Tadokoro (3, 4) stated that the brains of male animals were richer in lecithin, those of females richer in sphyngomyelin and galactolipids. According to Block (5) the aminoacids of the brains of both sexes are about the same, except the relation of lysine to arginine which in primates is 100:83 for the male and 100:93 for the female.

The present investigation is concerned mainly with the values for the different lipids of the brain of the white rat in continuation of previous work on the influence of the thyroid (6). White rats from our laboratory stock were used which had been inbred for the last 4 years. Altogether 12 litters were used with a total of 37 males and 49 females. Nineteen male rats were castrated and 29 females were ovariectomized at an age of about 52 days (±8 days) and allowed to survive for 120 days. The feeding and the methods used for the chemical investigation of the brain were the same as those described in a previous publication (6).

For the statistical evaluation of the results the mean values and their standard deviations were calculated. However, a more reliable comparative index is the percentage deviation of the operated animal from the non-operated control of the same litter. Figure 2 presents the mean values of all the individual percentage deviations. The fact that all the values for castrates have a minus deviation and those for the spayed rats have a plus deviation allows the drawing of definite conclusions as to their differences in growth and chemical composition of their brains.

DISCUSSION

The different behavior of castrated and ovariectomized rats in regard to their body weight is illustrated in figure 1. While the former is 15% behind in weight as compared with the male control of the same age, the spayed female is 21% heavier than the female control. These figures compare with the results of former investigators in castrated animals as follows: Donaldson and Hatai (1), 16%; Hatai (2), 9%; Korenchevsky (8), 10%; Commins (9), 19%; Rubinstein and coworkers (10), 12%.

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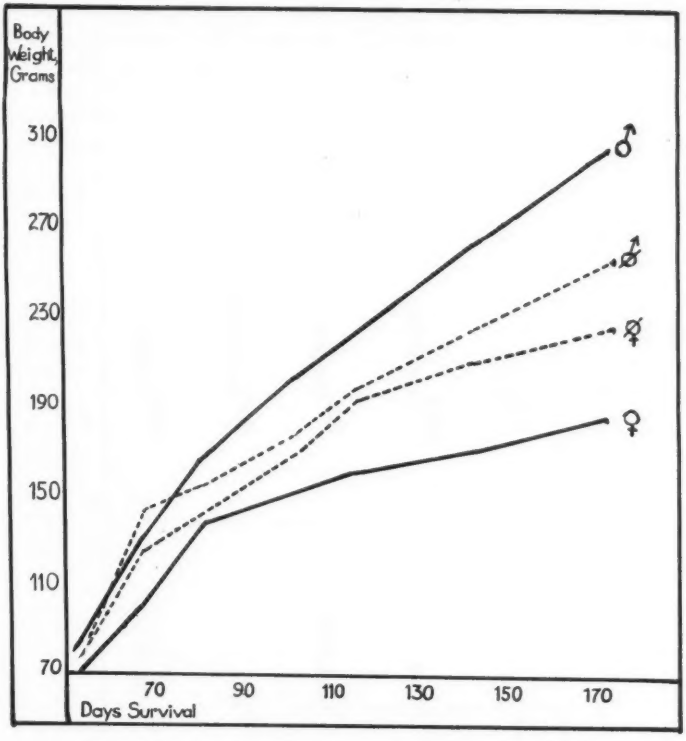

Fig. 1. Mean body weights of 18 male, 19 castrated, 29 ovariectomized and 20 female white rats.

The brain weight, too, remains smaller in the castrate animal (−18 per mille) and is increased relatively in weight (+47 per mille) in the spayed rat. These figures compare well with Hatai's results of −8 and +34 per mille respectively (2) (calculated

Table 1. Mean values and standard deviations together with percentage differences for the weight of body and brain. Survival 120 days

	m	c	c/m	f	o	o/f
No. of rats	18	19		20	29	
Original age, days	52	52		48	52	
Original wt., gm.	71 ±14	79 ±17		64 ±15	68 ±15	
Final wt., gm.	297 ±26	253 ±14	−15	184 ±9	223 ±13	+21
Increase, %	316	221	−29	188	228	+21
Brain wt., mg.	1807 ±45	1775 ±30	−18	1674 ±24	1755 ±45	+47
Per mille of body wt.	6.1	7.0		9.1	7.9	

m, male
f, female
c, castrate
o, ovariectomized

$c/m = \frac{c-m}{m}\ 100$ $\quad o/f = \frac{o-f}{m}\ 100$ (Multiplied by 1000 for brain weight)

from Hatai's figures for animals operated between 16 and 30 days of age). No comparative figures are available for the chemical constitution of the different brains, except for the water content (2), the different values of which have been analyzed above. Again, all the quantitative determinations of the different lipids, their phosphorus and calcium content show minus deviations for the castrate and plus deviations for the spayed rat (table 2, 3). A qualitative analysis, however, shows a close similarity in the chemical constitution of the 2 operated groups in comparison with their normal controls and of the control males and females among one another. There is one important

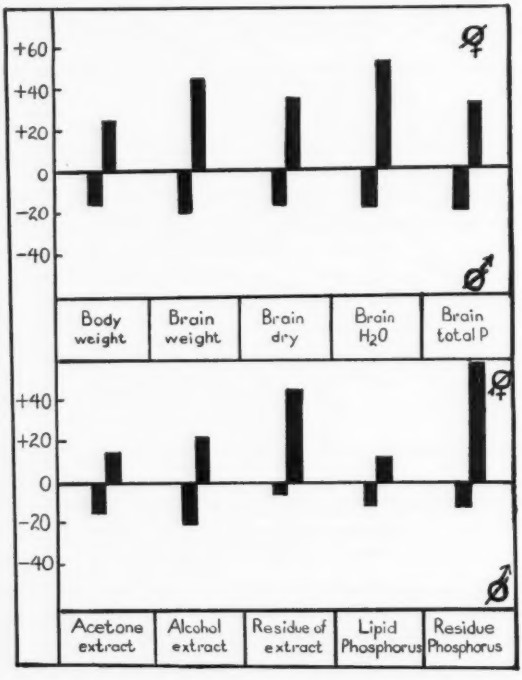

Fig. 2. Difference in the weight of body and brain and of the chemical composition of the brain of castrated and ovariectomized rats as compared with normal males and females respectively. For the body weight the difference from the normal control is calculated in per cent, for all the other figures in per mille of the corresponding normal values.

difference in the chemical constitution of the female brain in comparison with the male control, which is well beyond the limitations of error of the method employed, namely the higher percentage of the phosphorus of its alcoholic extract (2.42 and 2.26% respectively). In this one instance the ovariectomized animal shows a minus deviation from the female control (2.35% P), but again deviates toward the male average.

An analysis of the acetone precipitates from the different alcoholic extracts, containing the main lipids (table 4) indicates that this relatively larger content of phosphorus apparently belongs to that lipid fraction which is not precipitated by acetone. Furthermore, the brains of the normal females as well as those of castrated and spayed rats contain relatively more galactolipids and sphyngomyelin than the normal male brain.

TABLE 2. FRACTIONATE EXTRACTION OF THE LIPIDS OF THE BRAIN. METHOD OF H. AND I. MACLEAN (7). MEAN VALUES AND STANDARD DEVIATIONS TOGETHER WITH PER MILLE DEVIATIONS

	m	c	c/m	f	o	o/f
Dry wt., mg.	424 ±21	417 ±19	−16	392 ±12	406 ±17	+36
Water, mg.	1383 ±31	1358 ±24	−18	1282 ±27	1349 ±51	+52
% of brain wt.	76.5	76.4	− 1	76.4	76.7	+ 4
Acetone extract, mg.	68 ±2	67 ±3	−15	62 ±4	63 ±3	+16
% of dry wt.	15.9	16.0	+ 6	15.9	15.6	−19
Alcohol extract, mg.	143 ±10	140 ±8	−21	131 ±4	134 ±5	+23
% of dry wt.	33.7	33.6	− 3	33.3	33.0	− 9
Residue of extract, mg.	215 ±5	214 ±6	− 5	199 ±7	208 ±8	+45
% of dry wt.	50.4	50.4	0	50.8	51.4	+12

Despite the minus deviations of all the important figures for body and brain of the castrated and of the corresponding plus variations of the ovariectomized animal, they both never become numerically equal. This means that though both approach an

TABLE 3. PHOSPHORUS AND CALCIUM ANALYSIS OF BRAIN. MEAN VALUES, TOGETHER WITH PER MILLE DEVIATIONS

	m	c	c/m	f	o	o/f
Acetone extract, mg. P	0.74	0.73	−14	0.68	0.74	+88
% of extract	1.10	1.10	0	1.09	1.17	+73
Alcohol extract, mg. P	3.23	3.19	−12	3.16	3.15	+ 3
% of extract	2.26	2.28	+ 9	2.42	2.35	−29
Residue mg. P	2.08	2.01	−34	1.89	2.02	+69
% of residue	0.96	0.94	−21	0.95	0.97	+21
Total P, mg.	6.05	5.93	−20	5.73	5.91	+31
% of dry weight	1.43	1.42	− 7	1.46	1.46	0
Total lipid P, mg.	3.97	3.92	−13	3.84	3.89	+13
Calcium, mg.	0.36	0.34	−56	0.37	0.42	+135
Ca per 100 gm. of dry wt.	84	79	−60	94	98	+43

'asexual,' intermediate stage, yet the castrate still remains more 'male' and the spayed rat remains more 'female,' both in respect to the weight of body and brain and the chemical constitution of the latter. It should be interesting to discuss these findings in the light of our present conception of the correlation between the internal secretion

TABLE 4. COMPOSITION OF THE ACETONE PRECIPITATE FROM ALCOHOLIC EXTRACTS, CONTAINING MAINLY LECITHIN (ALCOHOL-SOLUBLE), CEPHALIN (ETHER-SOLUBLE), GALACTOLIPIDS (PYRIDINE-SOLUBLE) AND SPHYNGOMYELINS (RESIDUE). METHOD OF H. AND I. MACLEAN (7)

	Precipitate		In % of precipitate			
	% of alcoh. extract	% P	Alcohol-sol.	Ether-sol.	Pyrid.-sol.	Residue
Male	19.1	2.20	20.4	58.3	17.2	4.1
Castrate	26.8	2.00	19.5	47.9	26.2	6.4
Female	19.0	2.05	24.3	48.5	21.3	5.9
Ovariectomized	23.0	2.05	19.7	53.1	22.0	5.2

of the sex glands and the somatic sex characteristics. Previously, I had compared the extremely opposite opinions of Halban and Tandler-Grosz (11). The former claimed that male, female and hermaphrodite anlage is predetermined at the moment of fertilization of the ovum, while the latter assumed that the non-specific common asexual anlage was transferred into the specifix sexual form under the influence of the internal secretion of testes and ovary respectively. Applied to our present problem of body and brain weight and chemical constitution as secondary sex characteristics, it appears from a study of figures 1 and 2 that even after prepubertal removal of the sex glands there still remains a differentiation between castrated and spayed rats. This difference may be represented numerically by the difference in weight at the end-point of both growth curves. One may consider this difference as an expression of the determination of sex which takes place at the fertilization of the ovum and which is dependent upon the transmission of chromosomes. The difference between the male and the castrate on the one hand and between female and spayed rat on the other would indicate the specific influence of the internal secretion of testes and ovaries upon growth, either by direct action upon the soma or, indirectly by way of hypophysis, thyroid and suprarenal glands. The deviation of the spayed rat from the normal female is relatively larger than that of the castrate from the male control, as indicated in figure 2.

SUMMARY

The growth of body and brain and the chemical constitution of the brain were studied in normal male and female white rats and in castrated and ovariectomized animals.

Following the removal of the sex glands the weight of body and brain of male rats decreases and that of the operated female rats increases when compared with the normal controls. The same applies to the quantitative figures for the different chemical compounds of the brain. The qualitative composition of the brains of the 4 groups, however, is essentially the same, with the exception of a relatively higher amount of phosphorus in the alcoholic extract of the female brain and a higher percentage of the acetone precipitates of the alcoholic extracts of the 2 operated groups. Besides, the male brain is different from the 3 other groups by a relatively larger amount of ether-soluble lipids of the acetone precipitate of the alcoholic extracts (cephalin) and by a smaller amount of galactolipids and sphyngomyelin.

After the prepubertal removal of the sex glands there still remains a 'sexual' difference between castrated and spayed animals. The respective minus and plus deviations from the normal controls do not become the same numerically. It appears that the deviation of the ovariectomized animal towards an 'asexual' intermediate level is more pronounced than the deviation of the castrated animal from the normal male.

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THE INTERPRETATION OF THYMUS BODIES

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THYMUS BODIES which occur in all vertebrates, with the probable exception of the cyclostomes, remain today as enigmatic as when first examined, and this despite the great amount of work, morphological and physiological, observational and experimental, which has been devoted to their interpretation. Hammar (1) states that in the last 25 years more than 1000 publications have appeared devoted to the thymus. Certainly in an earlier period of 100 years nearly an equal number of publications[1] have demonstrated the interest centered in the thymus. Indeed, its large bulk in the young of man and mammals generally forced the query as to its nature and significance.

With new technics and experimental approaches, the conceptions of the thymus have shifted in accordance with changing theories, and the accumulated results have been summarized from time to time in reviews. First among these must be mentioned "Fifty years of thymus investigation" by Hammar (2) which, although it appeared over 30 years ago, still remains today a classical work of significance for any attempt to elucidate the thymus problem. Of more recent reviews, within the last four years, may be mentioned those of Gudernatsch (3), Hammar (1) and Nelson (4).

If these reviews are themselves analyzed, it becomes at once apparent that they are based on the tacit assumption that the thymus is an organ, a gland possessing a function, however obscure it may be—namely that it forms some product or products as a result of its metabolic activity which specifically contribute, directly or indirectly, to the maintenance of bodily unity. However, on examining in detail the results as presented by these surveys, it is clear that the evidence is still purely negative. All three of the reviewers discuss in detail the rather startling results published by Rowntree and his collaborators which describe marked effects on growth and differentiation attained through the injection of thymus extract. However, Hammar points out (p. 52) that several unsuccessful attempts have been made to obtain similar results by Rowntree's methods. Nelson comments that "attempts have been made in at least two other laboratories [in addition to his own] to confirm Rowntree's results. These results have been uniformly negative." Segaloff and Nelson (5) have published additional experimental results of a negative character. They also (6) repeated the earlier experiments of Asher (7) with thymus extract ('thymocrescin') but with negative results. It should of course be appreciated that a method of extraction can afford only circumstantial evidence, since it by no means follows that the substances thus procured are the same as those which the structure in question might give off as end products of its intrinsic metabolism. The most that may be safely expected is some intimation of the chemical composition of the structure concerned, and it is not surprising that the investigators of the effects of thymus extract have become largely concerned with the composition of the extracts themselves. Such analyses appear to be far from complete; however, the Asher extract appears to contain polypeptides and amino acids, at least tyrosine and histidine, and probably also cystine or cysteine.

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[1] No attempt has been made to provide a comprehensive bibliography of thymus work. The literature cited in the articles referred to will furnish all pertinent discussions.

The Rowntree extract is reported as containing ascorbic acid, glutathione and cysteine (8). It may be noted that through a long series of feeding experiments Gudernatsch (3) has shown that all of the amino acids mentioned above support growth, or growth and differentiation. However, in view of the unquestioned wide occurrence of these amino acids in body protein, their presence in the thymus and in thymus extracts affords no adequate evidence for a specific thymus function; as is in general recognized by the reviewers mentioned above. Hammar (1) in recent publications has particularly concerned himself with the vitamin content of the thymus. Vitamin C, ascorbic acid, as is well known, is abundant in the thymus, and may be demonstrated histologically in the original epithelial component (Hammar); whether formed there or stored there is not clear. Hammar also presents experimental evidence for the presence in the small thymocytes, an originally mesenchymal component, of a vitamin B, presumably thiamin. Both of these vitamins seem to be significant for growth, but as in the case of the amino acids considered, their wide distribution in the body gives no indication of a specific thymus function mediated by them. The presence of vitamin C within the thymus suggests an intimate association of the thymus with immunity. This essentially has been for some time a contention of Hammar (9) as is of course well known. But again, the obviously wide-spread occurrence of immunity reactions within the body does not support the view that therein is to be found a specific thymic function. Hammar (1) has indeed put the thymus problem succinctly when in his summary he states (p. 62): "I am convinced that all attempts to solve the question of the function of the thymus in one piece, so to speak, irrespective of the peculiar structure of the organ, can only meet with a rather incomplete success."

The above quotation from Hammar's review, doubtless refers to the peculiar structure of the thymus as an intimate intermingling of two tissues usually distinct, with perhaps an implication of two functions, distinct, correlated or antagonistic. However, in view of the prevailing negative results so far attained in the search for a function, the possibility—or even probability—should be entertained that the thymus possesses no specific function or functions. To most biologists this suggestion—that the thymus possesses no function—will seem absurd. The large size of the thymus body in the young mammal predisposes toward the assumption of some specific function. The general interpretative attitude is of course that the thymus is an organ, a gland, with a distinctive origin and a function presumably endocrine. Unfortunately for this assumption critical examination of the developmental evidence gives no support, and as has been indicated above, evidence for an endocrine function is at present entirely negative.

The term function itself calls for brief consideration. It is a term of obvious usefulness in biology but its value is qualified. We find it variously applied—to a muscle or a group of muscles, to an organ, to a cell or group of cells, or even to a definite chemical substance such as a vitamin. Its use implies a quality of action or effect which contributes directly or indirectly to the continuation or perpetuation of life. The term, function, implies performance and the teleologic aspect of its use is frequently quite distinct. It is more than convenient to recognize 'pattern' and 'process' as distinct aspects of life activity and its structural expression. To the former belongs the term function. Two further comments may be made; usually the structural pattern implies or suggests a function, but not always. A number of instances could be cited of definite structures obviously devoid of specific function. On the other hand it does not necessarily follow that the existence of a distinct function is always reflected in the structural pattern. Under this category would be the thymus—if indeed eventually it is shown to possess a function. It may be repeated that the thymus expresses an

atypical intermingling of two tissues usually distinct though intimately correlated—epithelium and connective tissue. The complex structurally offers no intimation of a gland of any type. It was this peculiarity of structure to which Hammar (1) referred in the quotation cited above.

The developmental processes which determine the thymic structure provide the basis for the interpretation of thymus bodies. It is fully recognized that the following is largely a repetition of facts, known though frequently disregarded. Briefly stated, the histogenesis of the thymus consists in an early invasion of the originally epithelial component by embryonic cells from the underlying and related mesoderm (mesenchyme). The epithelium at the same time usually loses its value as a surface tissue and undergoes a loosening or reticulation.

Both constituents of the complex undergo characteristic changes. The free cells from the mesoderm (mesenchyme) undergo a more or less marked proliferation producing cells structurally identical with the small lymphocytes of blood and lymph. At the same time the mesodermal constituent produces or provides, usually and typically, other features characteristic of connective tissue, namely, blood channels and other cell types with a certain degree of fibrogenesis. Usually these differentiations of mesenchymal origin are largely limited to the zone of the epithelial constituent bordering the mesenchyme (connective tissue). The well-known pattern of so-called cortex and medulla is thus determined. The epithelial component likewise undergoes a certain, but often marked, degree of proliferative growth attended frequently by a certain degree of cellular hypertrophy and frequently in the higher vertebrates, a central massing as cell conglomerates, the thymic (Hassall) corpuscles. A growth potentiality is thus evident in both thymic components. Where it is marked a lobulation, often extreme, is a result. In the typical mammalian thymus the concentration of the small thymocytes is apical in the lobule; the base where it joins other parts of the thymus is relatively free from them, and hence consists of medulla only. The thymic (Hassall) corpuscles, on the other hand are quite constantly central in the medulla of the lobule.

The thymic corpuscles have received a varied interpretation. The early review of Hammar (2) still remains the most comprehensive account of the occurrence, origin, structure and interpretations of these curious structures. The interest of Hammar in these structures and in the thymus problem in general has continued to the present time. In his monumental works (9, 10) he fully developed his view of a detoxicating function of the thymic corpuscles. Without considering the merits of this theory, it may be stated that, in the opinion of the present writer, thymic corpuscles are satisfactorily explained without the introduction of teleologic considerations. The thymus expresses fundamentally an epithelium which has lost its surface value but retains its growth potentiality and also its powers of differentiation which it typically expresses as a degree of keratinization. In such a surface epithelium the differentiation proceeds from the depths (connective tissue contact) toward the free surface; in the thymus lobule, the differential growth becomes centripetal, with cell conglomerates forming centrally as expressions of a differential growth in a confined space (11). Thymic corpuscles are thus similar in formation and structure to the epithelial pearls of a keratinizing epithelioma. Essentially identical formations may be expected, and are encountered, in any inclusion of a keratinizing epithelium within the connective tissue—as developmentally in the formation of the soft palate, or experimentally determined epithelial inclusions (12). Add to such inclusions a 'lymphocytic reaction' in the surrounding connective tissue with invasion of the epithelium and the structural pattern of the thymus is produced. In the thymus of the teleostome fish where the surface value of the thymic epithelium is retained (placoid thymus) (13) thymic

corpuscles are lacking—as would be expected. Typical 'thymic corpuscles' have recently been reported and figured in the palatine tonsil of the dog (14). The less typical epithelial formations of epithelial origin which are occasionally encountered (2) within the mammalian thymus (atypical, degenerating and cystic thymic corpuscles, epithelial cysts with stratified squamous, ciliated or mucous epithelium) are, from the developmental origin, quite comprehensible.

The comments made earlier in this article concerning the concept of the thymus as a gland may now be somewhat expanded; for quite generally the thymus is so conceived despite the fact that its structure gives no intimation of such significance; frequently as an endocrine gland of internal secretion whose function remains obscure. Sometimes it is viewed as primarily an original exocrine epithelial gland whose duct has been lost and hence it became endocrine secondarily—an interpretation applied also the the pituitary body and thyroid. As supporting this interpretation appeal is made to its development. Thus, in man, the thymus is commonly described as arising from a distinct primordium (anlage) in the pharyngeal entoderm appearing as a ventral out-pocketing of the third pharyngeal pouch, which 'migrates' into the thorax and there undergoes thymic transformation, its connection with the pharynx ('duct') becoming progressively attenuated and ultimately broken. Broadly considered, the embryology furnishes no support for such a speculation, nor indeed for the concept of the thymus as a definitive organ with a distinctive developmental origin. Comment may be made from several points of view. (*a*) The customary account of thymic development takes too little account of growth transformations and the mechanics of development. There is no active migration and the thymic cord is not a duct which disappears. The epithelium of the third pouch but shares in a relative displacement which characterizes the developmental transformation of the pharyngeal region of the embryo. Considering other mammals as well as man, it is evident that thymic development is variable. The thymic cord may in some forms itself undergo thymic transformation, in whole or in part. Also, but little if any thymic transformamation may take place in the entoderm of the third pharyngeal pouch. There may be little or no share in the relative shifting caudally which characterizes the pharyngeal transformations of development, and there results a cervical and not a thoracic thymus. (*b*) In certain mammals the ectoderm bordering the more caudal pharyngeal pouches (pouches three or three and four) mechanically buried as the cervical vesicle, undergoes thymic transformation. In most of the instances of cervical thymus the epithelial component is so derived. The guinea pig furnishes an example. (*c*) A certain but variable amount of thymus, referrable usually to the fourth pharyngeal pouch has been identified in a number of mammals. It is not found in rats and mice where a fourth pouch is absent or 'vestigeal.' The occurrence in man has been discussed recently (15). (*d*) In the lower vertebrates, although the epithelial component derives mainly at least from pharyngeal pouches (entoderm), the thymus bodies differ in number, location and distinctive origins not only as compared with the mammal but as between the different lower vertebrate forms. The matter is complicated by the tendency to view certain epithelial (epithelioid) buds from the pharyngeal epithelium as 'thymus' even though they disappear without undergoing thymic transformation. In view of a lack of any analysis of the mechanical and histogenetic factors in such cases, the value of the designation is questionable. It is hardly logical to term a structure a thymus which exhibits no characteristic features of a thymus. The branchiogenic concept which conceives each pharyngeal (branchial) pouch as possessing potentially a thymus primordium and possibly an 'epithelial body' (parathyroid) primordium is largely responsible. (*e*) Structurally it is often difficult to decide whether the designation thymus or tonsil is the more appropriate. It was the French histologist Jolly (16)

who first recognized that, considered broadly, tonsils and thymus bodies constitute an intergrading series of ascending structural complexity. He listed in such a sequence: Peyer's patches, tonsils, esophageal lymphoid organs of birds, the follicles of the bursa Fabricii of birds of prey, the lymphoid papillae of anal glands, placoid thymus of fishes, bursa Fabricii (of other birds) and the thymus of most vertebrates. Such structures Jolly termed "lympho-epithelial organs." In the first members of the above series, the epithelial component retains its surface value; in the last, typical thymus, this is lost. The list may clearly be extended, perhaps to include epithelio-lymphoid tumors. The fundamental community of structural pattern is paralleled by a common type of reaction on the metabolic side.

The metabolic characteristics of the thymus may be stated categorically as follows. The outstanding fact, well-known and recognized, is the marked sensitiveness of the thymus to conditions affecting the general body metabolism. This possesses two main aspects. Lowering the metabolic level causes marked diminution of the thymus affecting both components to a varying degree (accidental involution, Hammar). Restoration of the metabolic level is followed by an enlargement. Maintenance of a high metabolic level at least tends to be followed by an enlargment of the thymus beyond the average. It seems quite probable that the frequent enlargement of the thymus in cases of hyperthyroidism belongs in this category. The response of the thymus to metabolic level, it should be appreciated, is shared (as far as known) by other epithelio-lymphoid organs (e.g. tonsils) and by lymphatic tissue generally.

The second aspect links the thymus to the bodily growth as affected by the onset of reproductive maturity, and particularly with the gonads. It was known from an early period that the thymus diminishes in size after birth, but it was reserved for Hammar (10) to show that the diminution (age involution) of the thymus sets in approximately at puberty. In the slowing and cessation of growth, the thymus thus parallels quite closely the general bodily growth, and more particularly that of tonsils and lymphatic tissue generally. In the higher vertebrates at least, all three of these aspects of growth seem to express an alteration of metabolism linked with the onset of sexual maturity. The changes in the gonads may be suspected as rather directly responsible, and in the case of the thymus (and lymphatic tissue structures generally) the suspicion appears well substantiated. This need not be considered in detail here. The review of Nelson (4) may be consulted.

Reviewing the facts presented in the foregoing paragraphs, it is at once obvious that the concept of the thymus as a gland (or a group of glands) arising from a distinct primordium, receives no confirmation when thymic development is broadly considered. The attitude but reflects the prevalent 'organ concept,' which views the body as a complex of organs each with a distinctive origin and function (s).

All thymus bodies exhibit but one common feature, which Hammar (1) referred to in the sentence quoted above—namely, a characteristic intermingling of two tissues usually distinct, epithelium and connective tissue, or embryologically speaking, mesenchyme. In this peculiar interrelationship there is exhibited no suggestion of an adaptative morphology. The free cells from the mesenchyme show structural characteristics seemingly identical with the lymphocytes encountered in the blood and lymph. Such cells are quite widely encountered in the connective tissue. They are essentially an embryonic cell type and possess rather broad potencies as has been pointed out by more than one experimentalist. The epithelial component in the interrelationship exhibits no structural characteristics other than those which would be expected of an epithelium growing and differentiating in a confined space. However, the two components together seemingly possess a marked sensitivity to general body metabolism as expressed in growth and growth fluctuations.

It may be suspected that the epithelium in its atpyical relation to the underlying mesenchyme possesses an altered quality in its metabolism rather directly responsible for the characteristic interrelationship. Some years ago the author (17) suggested that the explanation of the peculiar epithelio-mesenchymal relation encountered in the thymus was to be found in the loss of the adaptative significance of the epithelium as a surface tissue. Thymic transformation would thus have an element of a "reaction of degeneration" and the fate of homiotransplants as summed up by Loeb (18) was referred to as circumstantial confirmation. Identification of the factor (or factors) directly responsible for the thymic differentiation is at present impossible. The term, thymus inducing potency—a mask of ignorance—may be used to express the unknown metabolic quality in the thymus-forming material. Such thymus inducing potency clearly may be present in varying degree, with a result that, when sufficient material is examined, a gradation of thymic reaction, slight to marked, will be encountered (compare Kingsbury, 19). This is particularly true of the 'thymic cord' and the cervical vesicle—material which in some mammals quite constantly, in others occasionally, undergoes thymic transformation. In general it would be expected—and indeed as far as observation has extended this is confirmed—a variable fate for such included epithelial structures will be found, namely: (*a*) disappearance without trace, (*b*) cystic persistence, the related connective tissue exhibiting varying degrees of a lymphocytic reaction or a fibrosis, (*c*) thymic transformation, typical or atpyical, marked or slight. As far as man is concerned, thymic transformation of the thymic cord is occasionally encountered. Doubtless sometimes it gives rise to cysts. The cervical vesicle seems usually to disappear[2] without trace, although occasionally it persists as a cyst calling for surgical interference. No instance of thymic transformation of the cervical vesicle in man is known to the writer. From conditions in other mammals, this may be expected, though rarely. To obtain adequate data the careful examination of a great amount of material would be required; a laborious procedure which few would care to undertake.

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[2] It has recently been claimed by Norris (20) that in man the cervical vesicle furnishes the developing epithelial component with a 'cortex' from which the thymic corpuscles are derived. From the epithelial component (entoderm of the third pharyngeal pouch) Norris derives the cytoreticulum. The thymocytes he recognizes as coming from the mesenchyme. Critical examination of his figures and description furnished the writer no evidence for his interpretation of the source of the thymic corpuscles. Since he ignores the mass of contrary evidence accumulated since the early observations of Hammar and of others, it is safe to say that the contention of Norris rests upon faulty observation and interpretation.

NOTES AND COMMENTS

RATE OF OXYGEN CONSUMPTION IN THE PARATHYROIDECTOMIZED ALBINO RAT

THE OBJECT of this study was to determine the rate parathyroidectomized albino rats use oxygen as compared to normal ones.

Apparatus. The apparatus consisted of a large water bath thermostatically controlled at 28° C. into which a modified Krogh-type spirometer and oxygen chamber were almost completely immersed. The oxygen chamber contained a small animal compartment to which one inlet conveyed oxygen from a tank and another led to the spirometer. A small weight counterbalanced the spirometer so as to produce a steady flow of oxygen. Trays of soda lime absorbed the carbon dioxide and moisture given off by the animal.

The animal chamber was made by arching thick celluloid over a hard rubber block. The ends were closed by perforated aluminum gates. Thermometers were kept in the water bath and in the oxygen chamber.

The animals were Wistar strain. A fast of 12 to 18 hours was enforced before every basal test.

Results. Most animals became accustomed to the small quarters in from 10 days to 2 weeks. Each animal was allowed at least 2 days between tests.

Satisfactory basal determinations were made on 26 of the 32 rats used. The parathyroids were removed from 26 and a small amount of thyroid from the others. From 4 to 12 tests were made before operation and 4 to 6 tests after operation. Of the 26 animals, 16 were not affected by removal of parathyroids. In 10 animals the rates of oxygen consumption were slightly affected: 6 irregular, 2 increased and 2 decreased. So far as could be determined, the removal of a small amount of thyroid did not affect the basal metabolic rate.

Discussion. Most of the rats in this series belonged to 3 litters. Each animal is a control because several tests were made before the parathyroids were taken out. A small amount of thyroid was taken out of the controls because it is impossible to remove the parathyroid without also excising a small amount of thyroid tissue. In every case, all tissue removed was sectioned to show completeness of operation and to be able to compare the amount of thyroid tissue removed from the controls with that removed when the parathyroids were taken out.

Summary. Consistent basal metabolic determination can be established on most adult albino rats. Removal of the parathyroid glands does not alter the rate of oxygen consumption in a significant amount in 26 albino rats. These tests were carried out over a period of 6 months.

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INFLUENCE OF THYROIDECTOMY ON MARE SERUM HORMONE ADMINISTRATION TO IMMATURE RATS

STUDIES in this laboratory[1] have shown that the gonadotropic response to prolan was not greatly influenced by divided dosage or thyroidectomy, in contrast to the response to the pituitary gonadotropic hormone, which response was markedly influenced by divided dosage or thyroidectomy. Under conditions in which the pituitary hormone was administered so that it was slowly resorbed and hence exerted its maximum effect, thyroidectomy was without influence. It was concluded that when thyroidectomy exerts an augmentation effect, the phenomenon could be explained by the decrease in rate of exchange in body fluids which this procedure brings about so decreasing the rate of resorption of hormone. If this contention is correct then the response to mare serum hormone, which is not affected to any extent by delayed resorption, should not be appreciably affected by thyroidectomy. Data originally presented by Evans et al.[2] give no indication of an augmentation to mare serum action in thyroidectomized rats. However, recently Smelser and Levin[3] state that the action of pregnant mare serum is considerably greater in thyroidectomized than in intact rats. In our opinion, this statement is not borne out by their ovarian weight data for the mare serum hormone: control ovarian weight 164 ±26; thyroidectomized ovarian weight 190 ±40. The increase of 17% is not statistically significant. On the other hand, the increase of 104% in the case of their hypophyseal preparation is significant.

We present the following data on the influence of thyroidectomy upon the response to mare serum injections (Cutter's Gonadin). Female rats were thyroidectomized at 21 days of age; 72 hours later they received 1 dose per day of mare serum hormone. This was repeated on 3 successive days. Autopsy was performed 96 hours after initial dosage. Litter-mate controls received the same dose of mare serum hormone.

Experiment		Body weight, gm.		Ovarian weight, mg.
		Start	End	
I	Intact rats (6)	44 ± 2.0	56 ± 1.7	38 ± 3.6
	Thyroidectomized rats (6)	37 ± 2.3	48 ± 1.7	60 ± 7.0
II	Intact rats (6)	47 ± 1.5	58 ± 1.4	63 ± 5.2
	Thyroidectomized rats (6)	41 ± 2.5	49 ± 1.7	74 ± 4.4

Since the body weight of the intact rats is 15 to 19% greater during the period of dosage than that of the thyroidectomized rats, the concentration of hormone in the intact rats may be considered to have been correspondingly less. A correction for this factor may be made using the mare serum assay data of Cartland and Nelson;[4] an increase in hormone administration of 15 to 19% over the amount required to produce 38 mg. ovaries (Experiment I) in intact rats would produce 52 mg. ovaries in intact rats, while the increase over the amount required to produce 63 mg. ovaries (Experiment II) would produce 79 mg. ovaries. A correction for body weight difference does not change deductions made for immature rats given prolan or the pituitary hormone.[1] In the case of prolan, ovarian weight increase with increasing dosage is much less than is ovarian weight increase with increasing dosage of mare serum hormone. In the case of the pituitary hormone, augmentation by thyroidectomy is much greater than could be accounted for by body weight differences. In adult rats thyroidectomized at 22 days of age body weight difference is great and has been considered in interpretation of data.[5]

It may be concluded that if dosage is proportional to body weight, thyroidectomy is without influence upon the gonadotropic response to mare serum hormone.

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GONADOTROPIC ANTAGONISM IN MATURE RATS

THE SO-CALLED ANTAGONISM PHENOMENON which is elicited when pituitary gonadotropic extracts are rapidly resorbed,[1,2] and which is manifested by inhibiting or decreasing the effects of gonadotropic agents in general has with one exception[3] been studied in immature test animals. Evans et al.[3] state that, "the effect of the antagonist in the normal adult female rat is distinctly different from that in the immature rat. Here excessive luteinization is observed, the ovaries being increased in weight and being composed almost entirely of corpora."

We are presenting data for 3 experiments in which mature female rats received the same pituitary gonadotropic preparation[1] which produced the antagonism effect to a high degree when administered to immature rats in soluble form either by the intraperitoneal or subcutaneous route. The purpose of the experiments was to ascertain whether antagonism would result against endogenous hormone. Injection was made once daily for 4 days. The rats were killed 96 hours after initial dosage. A comparison between litter mates was made in each experiment. In Experiments I and II, the rats were 68 days old the day of autopsy. In Experiment III, they were 90 to 100 days old.

Experiment No.	Total dose hormone per rat	No. of rats	Route of administration	Body wt., av.	Ovarian wt., av.	Uterine wt., av.
	mg.			*gm.*	*mg.*	*mg.*
I Control		6		153 ± 5	51 ± 5	180 ± 25
Dosed	2	6	Subcutaneous	150 ± 7	53 ± 5	99 ± 9
II Control		7		143 ± 5	43 ± 6	144 ± 20
Dosed	2+Cu	7	Subcutaneous	130 ± 6	85 ± 9	197 ± 29
III Control		7		186 ± 4	64 ± 6	294 ± 11
Dosed	3	7	Intraperitoneal	171 ± 8	67 ± 12	153 ± 9

It will be noted that in Experiment II, in which the hormone was administered in copper combination to delay resorption, there was a marked increase in ovarian weight. In each case the uterus of the dosed rat was larger than the uterus of its respective litter-mate control. In Experiment I, the same dose of hormone given in soluble form produced no change in ovarian weight, but produced a statistically significant decrease in uterine weight. The uterus of each dosed rat was smaller than the uterus of its respective litter-mate control. In Experiment III, in which the intraperitoneal route of injection was used there is no significant change in the mean ovarian weight. The standard deviation of the mean is, however, twice as large for the dosed group, illustrating considerable variation in response. The uteri are greatly reduced in size.

While these experiments fail to demonstrate antagonism against endogenous hormone as measured by ovarian weight decrease, they do demonstrate an inhibiting effect as measured by uterine weight changes. It should be pointed out that while 2 rats in Experiment III responded with marked ovarian hypertrophy, uterine weight was decreased while in Experiment II ovarian hypertrophy was accompanied by uterine hypertrophy in every instance.

On the basis of these experiments a warning note is offered as to what may be expected clinically when pituitary gonadotropic extracts are administered parenterally in soluble form: a suppression instead of stimulation of ovarian hormone secretion. Only multiple divided doses or the administration of an insoluble compound may be expected to stimulate ovarian secretion. It is suggested that the inertia which has developed in regard to the use of pituitary gonadotropic extracts in clinical trial may be due to the failure to produce a response with the pituitary extracts as conventionally administered.

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RELATIONSHIP OF VITAMIN A AND ESTROGENS

In the May issue of this journal Burrill and Greene[1] reported that administration of relatively large doses of vitamin A to rats produces no effect on the normal estrous cycle nor does it modify the action of estrogens upon the vaginal epithelium of castrate rats. These findings stand in opposition to those of Sherwood and collaborators[2] who found that feeding large amounts of carotene suppressed the normal estrous cycle.

Last year we conducted experiments similar to those of Burrill and Greene but circumstances prevented their completion. However, they were carried to a point where we can state that we arrived at tentative conclusions similar to those of Burrill and Greene. In one set of rats (Sherman strain), after castration we maintained the animals in continuous estrus with minimal amounts of estradiol benzoate, injected subcutaneously. Then, simultaneous daily subcutaneous injection with vitamin A in sesame oil, 10,000 to 40,000 I.U. per day for 2 weeks, had no demonstrable effect upon the vaginal smear. Control animals were injected with sesame oil. A second set of normal females, whose normal estrous cycle was determined, were fed daily over a period of 3 weeks 0.2 cc. of sesame oil containing 2,000 I.U. of vitamin A. Control animals received 0.2 cc. of sesame oil. These additions to the stock diet produced no change in the estrous cycle.

These results, confirming the report of Burrill and Greene, are described here in brief form for they indicate similar results with a different strain of animals, by parenteral as well as oral administration, and with different brands* of substances.

It is hoped that these experiments may be continued, and that the discrepancy between the findings of Sherwood and his collaborators, and those of Burrill and Greene and ourselves may be explained.

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* Vitamin A supplied through the courtesy of Dr. S. M. Gordon of Endo Products, Inc. Progynon B supplied through the courtesy of Dr. Max Gilbert of the Schering Corp.

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